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## GENETIC VARIABILITY AND GENE FLOW OF THE FALL ARMYWORM *SPODOPTERA FRUGIPERDA* (J.E. SMITH) IN THE WESTERN HEMISPHERE AND SUSCEPTIBILITY TO INSECTICIDES

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**GENETIC VARIABILITY AND GENE FLOW OF THE FALL ARMYWORM  
*SPODOPTERA FRUGIPERDA* (J.E. SMITH) IN THE WESTERN HEMISPHERE  
AND SUSCEPTIBILITY TO INSECTICIDES**

**By**

**Difabachew Belay Kondidie**

**A DISSERTATION**

**Presented to the Faculty of**

**The Graduate College at the University of Nebraska**

**In Partial Fulfillment of Requirements**

**For the Degree of Doctor of Philosophy**

**Major: Entomology**

**Under the Supervision of Professor John E. Foster**

**Lincoln, Nebraska**

**May, 2011**

**GENETIC VARIABILITY AND GENE FLOW OF THE FALL ARMYWORM  
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AND SUSCEPTIBILITY TO INSECTICIDES**

Difabachew Belay Kondidie, Ph.D.

University of Nebraska, 2011

Advisor: John E. Foster

The fall armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is an economically important pest of maize, sorghum, cotton, and rice in the Western Hemisphere. Previous studies on genetic diversity of FAW focused on identification of the corn and rice host strains; there is limited information about geographic genetic variation. To bridge this gap, I investigated the genetic diversity of FAW using representative samples from the United States, Argentina, Panama, and Puerto Rico with amplified fragment length polymorphism (AFLP). This study also investigated the susceptibility of the Puerto Rico FAW population to ten different insecticides used by Dow AgroSciences (DAS) Research Station, Puerto Rico.

Analysis of molecular variance (AMOVA) using AFLP revealed that the majority (71.2%) of the total variation is within FAW populations; only 28% of the variation was among populations. This indicates significant gene flow for FAW throughout the Western

Hemisphere. Also, cluster analysis showed the lack of regional genetic structuring. Moreover, there was no significant correlation between genetic dissimilarity and geographic distance, except for the Argentina samples, suggesting the presence of gene flow.

The FAW population in Puerto Rico remains susceptible to the insecticides used for its control. The insecticides Radiant, Orthene, and Larvin caused > 60% FAW mortality 16 h after application. Generally larval mortality increased with time after insecticide application; 96 h after application the majority of the insecticides gave > 80% control. Moreover, the dose rate study on selected insecticides showed that the current dosage used by DAS is sufficient to control the FAW.



## **Dedication**

**This Ph.D. dissertation is dedicated to my late father, Belay  
Kondidie.**

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## INTRODUCTION

According to the United Nations Food and Agricultural Organization (FAO) projection, the current six billion world population is expected to reach nine billion by the year 2050. World food price is increasing accompanied with increased demand for food crops which necessitates the need to increase food production in the coming decades. The years 2007–2008 saw dramatic increases in world food prices, creating a global crisis and causing political and economic instability and social unrest in different parts of the world. This food shortage is attributed to drought and increased oil prices that necessitates the increased use of biofuel, in part from maize which is a major world food crop.

Maize and rice are the most important crops in the world both for direct consumption and as raw materials for food processing industries. Maize is used for human food, animal feed, sweetener, biofuel, alcohol, and many industrial products (Martin et al. 2006). Maize supplies three-fourths of the nutrients derived from feed grain and over 80 percent of the silage fed in the United States. In 2006, maize ranked first with world production of 695 million metric tons followed by rice (634 million metric tons) and wheat (605 million metric tons) (FAO 2007). The top 5 producers of maize worldwide include USA (38%), China (20%), Brazil (6%), Mexico (3%), and Argentina (2%) (FAO 2007) and fall army worm (FAW), *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is one of the major insect pests causing substantial yield losses.

Although maize and rice are the major food crops of the world, their production is being constrained by insect pests like FAW among other factors. The problem is more complicated due to the presence of corn and rice strains of the pest in the Western Hemisphere where these crops are very important and produced at large scale.

FAW previously described as *Laphygma frugiperda* (S. and A.) (Vickery 1929, Wilson 1933) is native pest to the tropical regions of the Western Hemisphere from the United States to Central America and in the Caribbean to Brazil (Knipling 1980, Pashley et al. 1985, Pashley 1986, 1988b). The recognition of FAW as a serious economic pest dates back more than 175 years (Luginbill 1928). Since FAW does not survive conditions of prolonged freezing, in the United States it overwinters only in southern Florida and southern Texas (Barfield et al. 1980). A strong flier, it disperses long distances annually during the summer months. As a migratory and polyphagous economically important pest in the United States and Central America, it is capable of causing substantial losses in maize, sorghum, forage grasses, turf grasses, rice, cotton, and peanut production (Sparks 1979, Hall 1988). Although the FAW is known to feed on numerous plant species throughout the Western Hemisphere, it primarily feeds on corn, rice, Bermuda grass and other grass species and this polyphagous behavior has been attributed to dietary generalization (Luginbill 1928).

The FAW completes its life cycle in about 30 days during the summer. But, the duration can be extended to 60 days in the spring and autumn, and 80 to 90 days during the winter. There are no reports on the ability of FAW to diapause. FAW has 6 larval instars per generation and can have multiple generations per year (Capinera 2001). Adult FAW moths deposit a layer of egg masses on the leaves of host plants that will hatch within 2-3 days. FAW causes damage to crops mainly by larvae feeding on leaves of the plants. There are different options for controlling FAW including host plant resistance (conventional and transgenic), biological control and use of insecticides.

Previous studies on the genetics of FAW populations from the North America and Caribbean regions indicated the existence of two morphologically identical strains that differ in host preference, physiology, behavior, and pesticide susceptibility (Lynch et al. 1983, Pashley 1986, 1988a; Pashley et al. 1995, Prowell et al. 2004). One strain identified as the corn strain, mainly feeds on corn, sorghum, and other large grasses; the rice strain preferentially feeds on rice, Bermuda grass, and other small grasses (Pashley 1986). The two strains also differ in their mating behavior where the corn host strain mates in the first 2/3 of the night hours whereas the rice strain mates in the last 1/3 of the nights (Pashley et al. 1992).

Different molecular markers have been used to evaluate the genetic similarity and estimate gene flow among insect populations (Figuerola et al. 2002, Sosa-Gomez 2004, Martinelli et al. 2006, 2007). Various authors reported that the two host strains of FAW can be distinguished by strain-specific allozyme variants and genetic markers using different molecular techniques like polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and strain specific PCR (Pashley et al. 1985, Lu et al. 1992, McMichael and Prowell 1999, Levy et al. 2002, Meagher and Gallo-Meagher 2003, Nagoshi and Meagher 2003a). Nagoshi and Meagher (2004b) compared the genetic dynamics of the two host strains from corn fields before and after harvest and found that the corn strain constitute 72 and 39%, respectively. The authors also reported that samples collected from wild population consisted of more than 90% of the rice strain. Recently, Clark et al. (2007) analyzed the genetic diversity of the corn strain populations from Mexico, United States, Puerto Rico, Brazil, and Argentina and reported that the majority of the genetic variation was within population not between populations

indicating the presence of continuous gene flow between the different geographic areas where FAW was sampled. However, other studies have shown the presence of physiological and behavioral differences between populations of FAW that are consistent with reproductive isolation caused by geographical separation (Young 1979). Hence, it is still unclear the extent to which the dispersed populations of FAW in the Western Hemisphere genetically interact. Murua et al. (2008) observed differences in duration of different life stages, pupal mass, and reproductive characteristics among FAW population collected from different host plants and also within corn host plants. Studies in North America and South America FAW populations revealed two host strains that display similar host specificities in both geographic regions suggesting that either the divergence of the two host strains preceded the dispersion of FAW into North and South America or there is a significant gene flow between these geographically distant populations (Nagoshi et al. 2007).

Analysis of population genetic structure, i.e. the distribution of genetic variation within and among populations, is a key aspect to understand insect pest population dynamics in agricultural scenarios and understanding population structures provides the most fundamental information for reliable identification of species and design of management strategies. Moreover, understanding the genetics of pest invasion may help to identify the origin, the number of introductions and the spread of the infestation of a pest in an area. For example, comprehensive genetic analysis of different Mediterranean fruit fly (*Ceratitidis capitata* Wiedemann) populations has led to new findings about the source of invasion of the pest in California than it was thought before which helped in designing the area-wide management program using sterile male technique (Hoy 2003).



Despite the possible benefits that population genetic analysis of FAW may provide towards understanding dispersal, monitoring the spread of insecticide resistance, and implementation of area-wide management programs, relatively little information is available in this area. Most of the FAW research has been concentrated in identifying behavioral and physiological differences between the two strains and only few studies were done on geographic genetic variability using limited samples and few molecular markers. Hence, there is a need to study the spatial genetic variability of FAW within the same host strain.

In Puerto Rico numerous seed companies conduct both research and production activities on corn throughout the year. FAW is the most important pest; insecticides are sprayed 3 times a week during the peak season which places heavy selection pressure on the pest for development of insecticide resistance. This necessitates the need to conduct insecticide resistance monitoring. Therefore, the objectives of the present study were to investigate the genetic diversity and gene flow of FAW in the Western Hemisphere by analyzing large sample size and molecular markers and to assess susceptibility of Puerto Rico FAW population to insecticides being used by Dow AgroSciences Research Station, Santa Isabel, Puerto Rico. The results of the present study are important for the development and implementation of FAW management strategies including deployment of transgenics, insect resistance management, chemical control, and area-wide management programs.

**CHAPTER 1**

**DISTRIBUTION, BIOLOGY, CONTROL AND GENETIC**

**VARIABILITY OF *SPODOPTERA FRUGIPERDA* (J.E. SMITH)**

# 1. DISTRIBUTION, BIOLOGY, CONTROL AND GENETIC VARIABILITY OF

## *SPODOPTERA FRUGIPERDA* (J.E. SMITH)

### 1.1 FAW and related species

The order Lepidoptera, one of the largest insect orders in the world and contains butterflies and moths. Butterflies and moths are characterized by scales on their wings that come off when they are handled. Many species in the order Lepidoptera are economically important pests feeding on plants, stored grains or fabrics. Insects that belong to the order Lepidoptera undergo complete metamorphosis passing through egg, larva, pupa and adult stages. The genus *Spodoptera* belongs to the family Noctuidae where most of the moths are nocturnal. Noctuidae larvae are smooth and dull colored having 5 pairs of prolegs; most of them feed on foliage of plant and few on fruits (Borror et al. 1989).

The genus *Spodoptera* consists of a number of species that are important crop pests including *S. littoralis* (Boisduval) (the Egyptian cotton leafworm), *S. exempta* (Walker) (the African armyworm), *S. litura* (Fabricius) (the tobacco caterpillar), *S. exigua* (Hübner) (the beet armyworm), *Spodoptera ornithogalli* (Guenée) (Yellowstriped armyworm), and *S. frugiperda* (J.E. Smith) (the fall armyworm). The larvae of the African armyworm are major pests of cereals and rangeland in many sub-Saharan African countries; during outbreaks, the species' population size and invasion areas can be vast.

## **1.2 Origin, distribution and migration of FAW**

The FAW is tropical to subtropical in origin in the Western Hemisphere (Luginbill 1928, Sparks 1979). It is a common pest of several crops in the tropics of North, South, and Central America including West Indian Islands which suggests that the pest is tropical in origin (Vickery 1929). FAW population has also established in Israel; genital analysis on samples from Israel, Brazil, the British Museum, and drawings from Luginbill (1928) suggested that the Israel population is originated from Caribbean and United States region, not from Brazil (Wiltshire 1977).

Although it is tropical in origin, FAW has become a permanent resident of the Southern Gulf coasts of the United States where it can survive mild winter climatic conditions. FAW has a migratory behavior with high dispersal capacity that allows the pest to quickly spread along the range of its host plants. In early spring, FAW moths emerge from the overwintering pupae and migrate long distances to areas where the climate permits their survival; this can occur for successive generation from spring to fall (Vickery 1929). However, they are unable to survive the winter in the northern states and the species is destroyed each winter throughout its range in the United States except in the southern part where the winter is mild.

According to Vickery (1929), no direct evidence of FAW migration exists but its inability to overwinter in the northern United States and its annual appearance in those states indicates migration of FAW. Moreover, recent molecular works confirmed that the genetic variability of the pest in the Western Hemisphere, including northern and southern states of United States, is minimal indicating the presence of migration (gene flow) in this species (Clark et al. 2007). It is also suggested that part of the early spring

infestation in the southern United States may be caused by moths that migrate from the tropics (Vickery, 1929). South to north migration of the FAW seems to occur every year; however large outbreaks occur in occasional years.

Nagoshi and Meagher (2004a) mentioned that population surveys in southern Florida corn fields typically show a rise in the overall fall armyworm population in the spring, followed by a rapid and prolonged decline during the summer months that presumably reflects the northward annual migration of the pest. After the summer decline in the south, fall armyworm populations begin increasing in the fall and winter in agricultural areas, coincident with the late year corn growing season. The timing of this increase was shown to correlate with weather and wind conditions conducive to southward migration, leading to the suggestion of a north-to-south return movement prior to the winter freeze (Pair et al. 1986, Mitchell et al. 1991). Seasonal monitoring of FAW using sex pheromones for two years at eight locations from French Guiana northward to Canada showed a seasonal progression of movement by fall armyworm from the southernmost locations in the United States into Canada (Mitchel et al. 1991).

Area wide management programs designed to cause changes in fall armyworm population dynamics in the overwintering areas can significantly alter the magnitude of the northward migration (Knipling 1980, Mitchell et al. 1991,). In addition to long distance migration, understanding movement of FAW between crop fields is also important to develop appropriate management strategy. Martinelli et al. (2006, 2007) observed a considerable gene flow between FAW populations collected from cotton and corn fields in Brazil. This movement of FAW between different fields and host plants needs stewardship of crop protection methods for managing FAW to reduce the incidence

of pesticide resistance due to the spatial and temporal overlapping of maize and cotton crops in some regions (Martinelli et al. 2007). Similarly Nagoshi et al. (2006, 2007) found that the FAW infesting cotton in Mississippi comes from corn and suggested that corn fields provide an important refuge for the FAW strain infesting cotton and that late season populations in the Mississippi delta may be migrants from more northern corn areas.

The species overwinters in southern Florida and southern Texas, which serve as sources of the spring time populations that migrate northward into the central and eastern United States and Canada (Barfield et al. 1980). This capacity for a long distance movement, up to 480 km/generation (Sparks 1986), has contributed to widespread distribution of the FAW in the Western Hemisphere (Nagoshi et al. 2007). This seasonal migration of FAW could occur in response to seasonal changes in rainfall, temperature, and planting of host plants. Moreover, prevailing winds and frontal systems with their converging air masses during the spring are thought to largely determine the extent and direction of FAW adult migration (Rainey 1979, Pair et al. 1986).

Studies on annual migration of fall armyworm in North America can be made by comparing chemical or viral susceptibility of fall armyworm populations from different locations (Fuxa 1987, Pitre 1988), monitoring adult moths by pheromone trapping and radar (Rose et al. 1975, Pair et al. 1987), and correlating trap collections with wind and weather patterns (Luginbill 1928, Pair et al. 1986, Westbrook and Sparks 1986). However, the resolution of these detection methods is very low and more accurate and efficient molecular techniques should be employed which can identify strain specific migration (Nagoshi et al. 2008). Haplotype analysis to study migration of FAW corn

strain populations in Louisiana, Mississippi, and Alabama were statistically indistinguishable from populations sampled in central and southern Texas suggesting the fall armyworm overwintering in Texas migrate north and eastward through Louisiana, Mississippi, and into Alabama, whereas Florida populations move northward into Georgia (Nagoshi et al.2008). Machado et al. (2008) studied Brazilian FAW populations collected from corn and rice and found that the sex-linked tandem repeat element called *FR*, which was previously shown to have a strain-biased distribution in North American populations, suggested presence of gene flow between the Brazilian and North American FAW populations.

### **1.3 Description and Biology *S. frugiperda***

Under favorable conditions, the FAW completes its life cycle in about 30 days during the summer. However, the duration can be prolonged to 60 days in the spring and autumn, and 80 to 90 days during the winter. Depending on the appearance of the migrating adults and climate, FAW can have multiple generations per year. According to Capinera (2001), FAW did not enter into diapause. Seasonal migration is a major factor in the life history of FAW and it is considered as one of the most mobile noctuid crop pests in the Western Hemisphere.

First records of the FAW in the United States dates back to 1797 in Georgia (Johnson 1988). The moths fly at night and during the day they hide in the unopened leaves of the host plants upon which the larvae feeds. It is suggested that the moths lay their eggs during the night but they may also lay during the day to some extent (Vickery 1929). Females may mate several times and use sex pheromones to attract males (Sparks

1979). Eggs are usually laid on the upper surface of the leaves but occasionally they may lay on other parts of the host plants. The egg of FAW is dome shaped with flattened base that measures about 0.4 mm in diameter and 0.3 mm in height. Eggs are laid in mass and number of eggs per mass can vary from 100 to 200. A single adult female can lay on average 1500 to 2000 during its life time. Earlier studies indicated that one female has laid 1,782 eggs in 13 masses (Vickery 1929). Eggs are mostly spread over a single layer attached to the foliage and sometimes can be deposited in layers. Females deposit a layer of grayish scales from their body between the eggs and over the egg mass giving a furry or moldy appearance to the egg mass. Eggs hatch within 2-3 days in summer months (Vickery 1929, Capinera 2001). The duration of the egg stages was found to be shorter in the laboratory than in the insectary at the same temperature (Vickery, 1929).

FAW passes through six larval stages with head capsule width of about 0.35, 0.45, 0.75, 1.3, 2.0, and 2.6 mm, for 1-6 instars, respectively. Length of larvae varies from 1.7mm to, 34.2 mm, for the first and last instar, respectively. During hatching, the larvae feed on the egg shells, and for a short period they feed in colony near the egg mass. Newly hatched larvae are gregarious and feed on the leaves of the host plant on which the eggs were deposited, but when they grow larger they will disperse to other plants (Vickery 1929). The first and second instars feed on one side of the leaf skeletonizing it, but as they grow they eat making a hole through the leaf. Up to the third instar, the caterpillars can hide between host leaves and there is not much cannibalism. However, at latter stages they will compete for the throat (whorl) area of host, like in corn, and only one larva will survive by killing others. Hence, in rearing experiments in the laboratory, it is necessary to isolate them to avoid cannibalism.

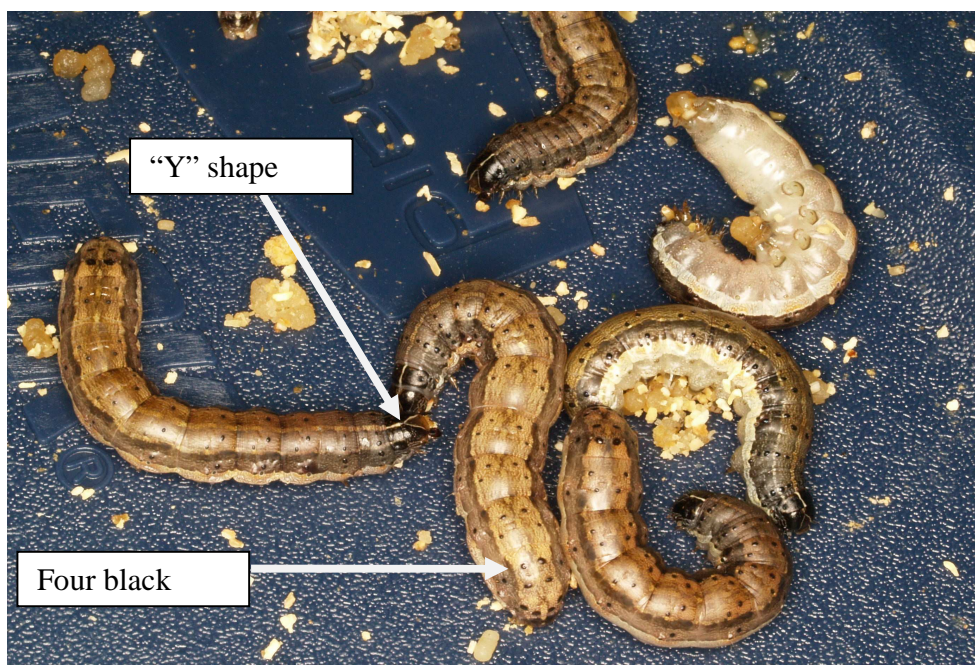


Newly hatched larvae of FAW are greenish with a black head, the head turning orangish in the second instar. At the end of the second larval stage, the dorsal surface of the body becomes brownish, and lateral white lines begin to form. Once attaining fourth and later instars the head becomes reddish brown spotted with white, and the brownish body bears white subdorsal and lateral lines. Dark and spiny elevated spots occur dorsally on the body. Matured FAW larva has a white inverted "Y" mark on its head and possesses a rough or granular texture when examined closely (Fig. 1). The four black dots at the last abdominal segment are also distinctive to FAW larvae. Larval stages last for about 14 days during the summer and 30 days during cool weather (Capinera 2001).

For each larval stage, there is an active feeding period and an inactive period which occurs before each molt. Although temperature can affect the length of both periods of the larval stage, lower temperature prolongs more the inactive period than the active period, and during the active period food supply is more important (Vickery, 1929). According to Pitre and Hogg (1983), when FAW is reared on maize at 25°C the mean development time was determined to be 3.3, 1.7, 1.5, 1.5, 2.0, and 3.7 days for instars 1 to 6, respectively.

Pupation of FAW normally takes place in the soil at a depth of 2 to 8 cm. Prior to pupation, the larva constructs a loose oval shape cocoon by tying soil particles with silk. In situations where the soil is too hard, larvae may web together leaf debris and other materials to form a cocoon on the soil surface. The pupa is 14 to 18 mm long and 4.5 mm wide in size, and reddish brown in color (Fig. 2). During summer, pupal period lasts for 8-9 days; in winter it can take up to 20-30 days (Capinera 2001) and may be as long as 55 days (Vickery, 1929). In FAW pupation experiment it was observed that with specimens

of both sexes pupating at the same time the females emerged a day or so earlier than the males (Vickery 1929).



**Figure.1.** Fifth instars of *S. frugiperda* with identification features.

Adult moths of FAW are variable in color and their wing span can reach 32 to 40 mm. Male moths have a shaded gray and brown forewing with triangular white spots at the tip and near the center of the wing. Forewings of females are less distinctly marked, ranging from a uniform grayish brown to a fine mottling of gray and brown. The hind wing of both sexes is shining silver-white with a narrow dark border. Adults of FAW are nocturnal and are most active during nights.



**Figure 2.** Pupae of *S. frugiperda*. Photo: Belay 2009.

After emergence, the adult female deposit her eggs during the first four to five days of its life, but some oviposition occurs for up to three weeks. Adult longevity is about 10 days on average with a range of about 7 to 21 days. In Texas during hot months of May-September, it will take 23 to 28 days to complete one generation (egg to egg) and in such situations there could up to six larval stages and 9 to 11 generation per year (Vickery 1929).

### **1.3.1 Host range**

The FAW is a polyphagous pest that attacks over 80 plant species, however, grasses are the most preferred hosts. FAW is observed to feed on a large number of host plants especially during an outbreak seasons when the caterpillars migrate from destroyed grasses to neighboring plants (Vickery 1929). It commonly feeds on field corn, sweet

corn, sorghum, Bermuda grass, rice and grass weeds such as crabgrass and *Digitaria* spp. Other field crops that are frequently injured by FAW include alfalfa, barley, buckwheat, cotton, clover, oat, millet, peanut, ryegrass, sugar beet, Sudan grass, soybean, sugarcane, timothy, tobacco, and wheat (Knipling 1980, Pashley 1986). In Venezuela, FAW is one of the most important crop pests feeding on 31 host plant species belonging to 21 families (Labrador 1967). Andrew (1988) also reported that in Ecuador, the species is a problem on maize, cotton, tobacco, tomato, cucumber, rice, sugarcane, beans, soy beans, various grasses and forage legumes.

When the FAW larvae occur at high density, they defoliate the preferred host plants, acquiring an "armyworm" habit and disperse in large numbers consuming nearly all vegetation in their path. However, hosts consumed during such periods of abundance of FAW are not indicative of oviposition and feeding behavior of the pest under normal conditions (Knipling 1980).

Among vegetable crops, sweet corn is the only host which is regularly damaged, but other vegetables are attacked occasionally including apple, grape, orange, papaya, peach, strawberry and a number of flowering plants. Weeds known to serve as hosts include bentgrass, *Agrostis* sp. crabgrass, *Digitaria* spp.; Johnson grass, *Sorghum halepense*; morning glory, *Ipomoea* spp.; nutsedge, *Cyperus* spp.; pigweed, *Amaranthus* spp.; and sandspur, *Cenchrus tribuloides*.

There are evidences for existence of two morphologically identical host strains of FAW that are defined by their host plant preferences (Pashley et al. 1985, Pashley 1986). One strain feeds principally on corn, sorghum, and other large grasses (corn strain), and

the other strain preferentially feeds on rice, Bermuda grass, and other small grasses (rice strain).

### 1.3.2 Damage

Female moths of FAW prefer young corn plants that are 1 to 2 feet in height for oviposition and the small caterpillars are found feeding on leaves of young corn plants. However, older larvae penetrate into the whorl (throat) and stems feeding day and night. Because of its feeding habit in the throat of corn plants, previously it was mistakenly identified as budworm (*S. exigua* Hbn.) (Vickery 1929). Larvae of FAW cause damage to host plants by consuming foliage and early instars consume leaf tissue from one side leaving the opposite epidermal layer intact. But the second or third instars feed on both sides of the leaf making holes.

Leaf feeding by FAW larvae usually starts from the margin and proceeds to the midribs of the leaves. When the larva feed on whorls of corn, clear perforations are observed on the leaves. Holes are formed due to feeding of folded leaves and when the leaves grow out, a row of three or four small to large holes are seen across the leaf. Usually larval densities are reduced to one or two larvae per plant as a result of cannibalistic behavior when larvae feed in close proximity. Feeding by older larvae causes extensive defoliation often leaving only the ribs of leaves and stalks of corn plants, or a ragged and thorny appearance.

Marenco et al. (1992) indicated that infestation by FAW on sweet corn causes more injury at late whorl stage compared to early and mid-whorl stages. Larvae of FAW burrow into the growing point of plants (buds, whorls, etc.) and destroy the growth

potential of plants, or clip the leaves. In corn, they also burrow into the ear and feed on kernels like that of corn earworm, *Helicoverpa zea* (Boddie). But, unlike corn earworm, fall armyworm will feed by burrowing through the husk on the side of the ear. Leaf damage by FAW and corn earworm is also confusing. However, it is possible to determine which species is responsible for the damage through close examination as the holes formed by FAW have smooth edges where as holes cut by corn earworm larvae have ragged edges (Vickery 1929).

### **1.3.3 Economic importance**

The FAW is a major agricultural pest in most parts of the Western Hemisphere extending from southern Canada to central Argentina (Ashley 1986). Presence of multiple generations, ability to migrate and feed on a wide host range makes FAW the most severe economic pest in the Western Hemisphere. In the United States, fall armyworm is a regular pest of corn, sorghum and turfgrass (Sparks 1979, Pashley 1988a, Foster 1989). During large outbreaks, the pest causes significant damage to cotton, and sugarcane (*Saccharum spp.*) (Hall 1988, Pashley 1988b). On average, yield loss caused by fall armyworm damage in the US is estimated to be 2% annually (Wiseman and Morrison 1981). In another study, Wiseman and Isenhour (1993) showed that commercial maize hybrids suffered a yield loss of 15.4 -32.4% when manually infested with 2 applications of 20 neonates per plant. In the United States in 2003 FAW was ranked to be the 8<sup>th</sup> most important insect pest of cotton at a national level and the 3<sup>rd</sup> most important pest to cotton in Arkansas for the same year (Williams 2003). In Arkansas, costs incurred to prevent yield losses in cotton due to FAW damage in 2003 were estimated to be \$2.7

million for insecticide treatments against it (Williams 2003). According to Sparks (1986), FAW ranks second among the most damaging agricultural pests in order of total losses, ranging from \$39 to \$297 million annually. Martinelli et al. (2006) also reported that the FAW is one of the primary pests of maize and cotton in South America.

It is also reported that FAW is the most destructive and economically important insect pest of maize fields in Brazil (Sena et al. 2003). Chemical control of FAW becomes more difficult due to development of insecticide resistance. Several reports indicated the existence of insecticide resistant strains of FAW (Pitre 1988, Yu 1991). The pest causes severe economic damage in corn and cotton fields in Brazil and the occurrence of the pest in both maize and cotton fields has complicated the implementation of integrated pest management programs in these two crops (Martinelli et al. 2006). Hence, in Brazil, use of insecticides is considered as the major component of IPM used by farmers and the over uses of insecticides has led to the development of resistance by the FAW.

Hruska and Gladstone (1988) reported that FAW is one of the prominent pests of maize in Nicaragua where the level of infestation reaches up to 100% and, if untreated, causing a 45% yield reduction. In Nicaragua, application of fertilizers in hybrid maize increased yield by only 6% but when insecticide application was added to control FAW, yield was increased by 60% indicating the importance of FAW as a crop pest (Van Huis 1981).

FAW outbreaks are attributed to the migration behavior and egg laying habit of the moths. The female moths seek young host plants including corn and, when young plants are available in areas where large numbers of moths are flying, they will

concentrate on those fields for oviposition producing large number of caterpillars thereby destroying the crop; that causes migration of the caterpillars in armies to other fields (Vickery 1929). FAW outbreak is more common in late planted corn as we have observed in Nebraska (Clay Center and Elkhorn) in 2008 during collection of FAW samples for this study.

The potential of FAW to become an economically important pest and occupy wider geographic areas is attributed to its ability to move long distance from overwintering sites to other areas of infestation. Since FWA does not enter into diapause, its appearance in areas with freezing winters arises from populations that overwinter in milder climates and migrate in the spring throughout the summer. Previous studies indicated that FAW infestation in central and eastern United States and southern Canada is from annual migration of overwintering population from Mexico, Texas, and Florida (Young 1979, Pair et al. 1987).

Despite its economic importance, FAW is difficult to manage because of its broad host range, wide geographic distribution, development of resistance to insecticides, rapid and long distance movement which can serve as an escaping mechanism from biocontrol agents that can help to regulate less mobile insect pests (Knipling 1980).

## **1.4 Control of FAW**

### **1.4.1 Monitoring and Sampling**

Detection of presence of a pest and estimating its population density is important for timing of management tactics. FAW monitoring can be done by capturing the flying moths with black light and pheromone traps. Pheromone traps are more efficient



compared to backlight traps; they should be suspended at canopy height at the whorl stage in crops like corn (Starratt and McLeod 1982). Trap catches can determine the presence or absence of the pest, however they are not necessarily good indicators of density.

Once moths are detected, it is advisable to search for eggs and larvae. Percent infestation can be assessed by sampling 20 plants in five locations, or 10 plants in 10 locations (Capinera 2005). Van Huis (1981) recommended that resource scarce farmers should count the number of injured whorls in 20 consecutive plants at 5 randomly selected sites. Similarly, Andrews (1988) recommended sampling 20 plants per site from 5 sites and use of a 40% infestation of maize as a threshold in Honduras. To determine larval density in a field, large sample size is needed, especially when larval densities are low or larvae are young. In pasture lands sampling is done by walking from four sides following the diagonals of sampling area using a square foot metal quadrant. If larval density per square foot is 3 or more, the threshold is reached to apply treatments (Flanders 1995).

#### **1.4.2 Cultural Control Methods**

Cultural control is an important component of pest management strategies including FAW. In the case of *S. frugiperda*, several studies have indicated that low or no till agriculture and polycultures are less attacked by the pest compared to monoculture cropping systems planted using conventional cultivation (Andrews 1988). In Cuba, intercropping of maize with sun flower resulted in lower infestation by FAW and higher yield compared to the maize monocrop (Ryder 1968). Del Rosario et al. (1981) in the

Dominican Republic showed that the use of no till techniques reduced whorl damage by FAW by 30-60% compared to the conventional planting.

Van Huis (1981) also found that in Nicaragua infestation of maize by FAW was 20-30% lower when interplanted with beans compared to planting maize alone. The mixed cropping systems are likely to support more predators, disrupt egg laying by FAW female moths and also hinders the plant to plant migration of FAW larvae after hatching. Leaving few strips of weeds between rows of maize also can help to reduce maize infestation by serving as unsuitable host for the larvae that move between maize plants. Flooding rice fields until the plants are nearly covered is a common practice in Venezuela in order to drown larvae (Labrador 1967).

### **1.4.3 Biological**

#### **1.4.3.1 Parasitoids and Predators**

Even though biological control may not replace conventional insecticides a number of parasitoids, predators and pathogens readily attack larval and adult stages of FAW. The migratory behavior of the FAW away from overwintering and reproduction sites makes the natural enemies less efficient as they are left behind. Hence, although FAW has many natural enemies, few act effectively enough to prevent crop injury. Study on classification and distribution of natural enemies of FAW by Ashley (1979) showed that 53 species of parasitoids representing 43 genera and 5 families attack FAW around the world. In Mexico, Molina-Ochoa et al. (2000) recorded 11 species of hymenopteran parasitoids from the families Ichneumonidae, Braconidae and Eulophidae that cause an average parasitism of 11.3%. The authors also reported that parasitism was higher in

areas with high diversity of parasitoids compared to areas with low parasitoid diversity. Similarly, Ruiz-Najera et al. (2007) conducted survey of parasitoids of FAW from 21 corn fields in Mexico and recorded overall parasitism rate of 20.1%. The parasitoids recorded include five braconids i.e. *Rogas vaughani* Muesebeck, *R. laphygmae* Viereck, *Chelonus insularis* Cresson, *C. cautus* Cresson, *Glyptapanteles militaris* Walsh, two ichneumonids i.e. *Neotheronia* sp., and *Ophion flavidus* Brulle, and one eulophid, *Euplectrus plathypenae* Howard. Dipteran parasitoids in the family Tachinidae are also reported to be important natural enemies of FAW in the region, contributing to 6.3% of larval parasitism (Ruiz-Najera et al. 2007).

Among parasitoids species that affect fall armyworm, *Cotesia marginiventris* (Cresson) and *Chelonus texanus* (Cresson) (both Hymenoptera: Braconidae), are the most commonly reared wasp parasitoids from larvae of FAW in the United States. Among fly parasitoids, *Archytas marmoratus* (Townsend) (Diptera: Tachinidae) is the most abundant (Capinera 2005). Vickery (1929) reported that nine species of hymenopterous parasitoid were reared from larvae of FAW, of which five were of the family Braconidea, three from the family Ichneumonidae and one species of family Eulophidae. Moreover, he reared two species of diptera that belong to family Tachinidae.

One important problem that affects efficiency of parasitoids is competition among them as the same individual host may be attacked by different species of parasitoids. In this case, if the less efficient species survives, its attack can serve to reduce the number of more efficient parasitoid species. Hence, it is possible that a large number of parasitoids attacking the same host may result in less rather than more control of that host. Van Huis

(1981) reported 16 species of parasitoids and 3 entomopathogens which caused an average of 35% larval mortality.

The predators of FAW are general predators that attack larvae of other lepidopterans. The most important predators of FAW include various ground beetles (Coleoptera: Carabidae); the striped earwig, *Labidura riparia* (Pallas) (Dermaptera: Labiduridae); the spined soldier bug, *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae); and the insidious flower bug, *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) (Capinera 2005). Among the vertebrate predators, birds, skunks, and rodents are important ones that feed on larvae and pupae of FAW. Pair and Gross (1984) reported 60 to 90 percent loss of pupae to predators in Georgia indicating the importance of predators in biological control of FAW. Van Huis (1981) also has identified seven reduviids and four pentatomids as predators of FAW from Nicaragua.

#### **1.4.3.2 Entomopathogens**

In addition to the parasitoids and predators, FAW is also attacked by a number of pathogens including viruses, fungi, protozoa, nematodes, and a bacterium that cause significant level of mortality in FAW population and help to reduce leaf defoliation in crops. Among the pathogens, *B. thuringiensis* (All et al. 1996) and nucleopolyhedrovirus (NPV) (Garrdner and Fuxa 1980) are reported to be the most prevalent and potent in natural populations. Escribano et al. (1999) reported that NPV strains isolated from Nicaragua (Sf-NIC) and United States (Sf-US) had the highest infectivity rates compared to strains isolated from Argentina. Vickery (1929) isolated a fungal pathogen *Beaveria globulifera* (Spegazzini) that cause a common disease in FAW larvae.

FAW samples collected from Mexico, Colombia, and Brazil displayed different susceptibility to pure preparation of the Cry 1B, Cry 1C, and Cry 1D toxins from selected Bt strains (Monnerat et al. 2006). The difference observed in toxin binding capacity of these three Latin American FAW populations was found to be correlated with the observed differences in susceptibility to the tested Cry toxins. Genetic variability of FAW should be taken in to account when developing insect pest management strategies including the deployment of Bt corns. For example in Puerto Rico, where most of the biotech companies grow their winter nurseries or test their transgenic corn varieties, FAW became a major problem and insecticides are sprayed up to 25 times for one crop cycle to protect maize varieties including some Bt transgenics (personal observation).

In a survey, Molina-Ochoa et al. (2003) recorded a 3.5 % FAW larval mortality in Mexico due to naturally occurring entomopathogens and parasitic nematodes. The authors recovered three species of entomopathogenic fungi representing two different classes, Hyphomycetes (*Nomuraea rileyi*, and *Hirsutella sp.*) and Zygomycetes (*Entomophthora sp.*) from FAW larvae, and additional two species of Hyphomycetes (*Metarhizium anisopliae* and *Beauveria bassiana*) from soil samples. Different species of entomopathogenic nematode were also isolated from the FAW larvae as well as from the soil (Molina-Ochoa et al. 2003). Application of two species of entomopathogenic nematodes, *Steinernema carpocapsae* (Weiser) All strain and *S. riobravus* (Cabanillas, Raulston and Poinar), for controlling prepupae of the FAW has resulted in a significant mortality (Molina-Ochoa, et al. 1999). Integrated use of entomopathogenic nematodes and resistant corn silks enhanced mortality of FAW which could be useful for integrated management of the pest (Molina-Ochoa, et al. 1999). Plant allelochemicals like maysin, a

luteolin-C- glycoside, when used with a nuclear polyhedrosis virus (NPV) reduced damage by FAW by about 50%, and doubled the mortality of FAW compared to the susceptible check (Hamm and Wiseman 1986). Molina-Ochoa et al. (1996) also found that that *S. carpocapsae* All strain and *S. riobravo* were the most pathogenic nematode species against 7day old larvae, prepupae and pupae of FAW, with the prepupae the most susceptible stage. Improved control of FAW in field and sweet corn at whorl stage was obtained with oil flowable formulation, granule or wettable granule application of EG1999, a variant of Bt strain developed by recombinant DNA technology from EG2348 (the active ingredient of the bioinsecticide Condor<sup>®</sup>) at whorl stage of the crop (All et al. 1996).

#### **1.4.4. Host plant resistance**

##### **1.4.4.1 Conventional**

As defined by Painter (1951), resistance refers to the amount of heritable qualities possessed by a plant that influence the degree of damage by the target pest. Host plant resistance is an important, ideal and sustainable option of pest management including FAW (Luginbill 1969). The resistance mechanism could be antixenosis (non-preference), antibiosis or tolerance (Painter 1951). Host plant resistance research against FAW in corn, sorghum, peanuts, bermudagrass and rice involved screening, developing and releasing of germplasm (Davis 1980). The existence of partial resistance (antibiosis and antixenosis) in sweet corn was mentioned by Capinera (2001) but it did not provide complete protection. Corn gremplasms that showed moderate resistance to FAW leaf feeding were identified by Williams et al. (1999) and used in corn breeding programs to

develop corn hybrids that are resistant to FAW and other lepidopteran pests. Van Huis (1981) reported that in Nicaragua some hybrid maize varieties were resistant to FAW compared to local varieties.

In an experiment conducted to develop FAW resistant maize lines through back crossing, Abel et al. (2000) reported that two out of the 15 lines evaluated showed resistance to FAW. In another study, Braman and Duncan (2000) evaluated 21 paspalums and 12 zoysiagrass for their resistance to FAW. Their results indicated that six zoysiagrass showed high levels of antibiosis resistance, and four paspalums exhibited reduced larval or pupal weights or prolonged developmental times. In Mississippi at least two maize varieties were identified to have both antixenosis and antibiosis effect on FAW larvae (Wiseman et al. 1996). Pashley et al. (1987a) reported that the two host strains of FAW showed different developmental rates when feeding on Tifton 292 Bermuda grass where the corn strain was less affected than the rice strain. Some rice cultivars are also reported to show resistance to FAW (Lye and Smith 1980, Pantoja et al. 1986).

Generally host plant resistance is sustainable and compatible with other tactics of pest control and if FAW larvae fed on foliage of resistant varieties of corn during the growing season, they will show reduced growth, prolonged developmental time, and they would be exposed to parasites and predators for a longer period of time (Molina-Ochoa et al. 1999). Eight inbred lines of corn have been released by USDA-ARS, Starkville, Mississippi, with antixenosis and antibiosis resistance to FAW larvae and the germplasms were incorporated into commercial seeds by Delkalb/Pfizer (Sparks 1986)

#### 1.4.4.2 Transgenic

Corn germplasms exhibiting a moderate level of resistance to leaf feeding damages by FAW have been identified and released (Williams et al. 1999); they have been used to develop FAW resistant corn hybrids. Recent attempts to develop FAW resistant corn hybrids have focused on developing corn hybrids with genes from *Bacillus thuringiensis* (Berliner) (Bt) that encode insecticidal proteins. Williams et al. (1999) evaluated maize hybrids with both native resistance and genes from Bt encoding insecticidal proteins under field and laboratory conditions. They found that hybrids with both native and Bt trans-genes showed resistance compared to hybrids that had only native genetic resistance or Bt encoding insecticidal proteins alone. Moreover, they observed that larvae that fed on leaves of hybrids with both types of resistance were significantly smaller than larvae fed on susceptible hybrids or hybrids with only one type of resistance.

In a study conducted to evaluate resistance of transgenic maize varieties Bt11 and MON810 (both Known as YieldGuard®, Monsanto Company, St. Louis, MO), against FAW and corn earworm, key lepidopteran pests of corn in southeastern United States, Buntin et al. (2001) reported that YieldGuard® consistently prevented whorl and kernel damage, and yield loss by FAW and corn earworm. Endotoxin in these events of Cry1Ab gene is expressed in vegetative and reproductive structures throughout the season (Armstrong et al. 1995, Williams et al. 1997). Storer et al. (2001) also reported that in North Carolina corn hybrids containing Bt11 or MON810 events reduced ear damage by corn earworm by 80% and reduced corn earworm emergence from Bt corn fields by 75%.



Moreover, Bt hybrids also stunted the growth of surviving *H. zea* larvae and delayed adult emergence by 6-12 days.

Buntin et al. (2004) evaluated the performance of Bt MON events alone and pyramided with MON810 for their ability to prevent whorl defoliation by the FAW under artificial infestation and natural ear feeding damage by the corn earworm. Their results indicated that all Bt events tested reduced fall armyworm whorl damage and ear infestation by corn earworm compared to the nontransgenic isoline. The authors also reported that pyramiding events did not improve control of fall armyworm whorl damage compared with single events. But, they generally did prevent more ear damage by corn earworm. Moreover, they found that MON84006 event singly and pyramided with MON810 had superior control of whorl-stage damage by *S. frugiperda* and ear damage by *H. zea* compared with MON810. The authors suggested that deployment of new events and genes could provide additional tools for managing the potential for insect resistance to Bt toxins.

#### **1.4.5 Insecticidal**

As it is true in many other insect pest species, insecticides are important management options in FAW control. Insecticides are applied against FAW to protect against losses in different crops and pastures. In the Southern United States insecticides are applied on sweet corn against FAW, often on daily basis when the corn is at silking stage (Capinera 2001). In Florida, fall armyworm is the most important pest of corn and insecticides are applied against FAW to protect both the early vegetative stages and reproductive stage of corn. High volume of liquid insecticide is required to obtain

adequate penetration and kill larvae feeding deep in the whorl of the plants. In situations where overhead sprinklers are used for irrigation, insecticides can also be applied in the irrigation water. Keeping plants free of larvae during the vegetative period can help to reduce the number of sprays needed at the silking stage (Foster 1989). Hence, sprays should be spaced evenly during the growing period instead of concentrating at silking period.

In Mexico, chemical control of *S. frugiperda* in maize is achieved by application of methyl parathion, chlorpyrifos, methamidophos, and phoxim, among other insecticides (Malo et al. 2004). Sex pheromone produced by female *S. frugiperda* moths has been shown to be a useful tool to monitor male populations and schedule insecticide application to manage the pest (Adams et al. 1989, Mitchell et al. 1989).

Although chemical insecticides can provide effective control of crop pests including FAW, control of FAW has been dependent on insecticides and as a result the pest has developed resistance to major classes of insecticides in several locations (Yu et al. 2003). Corn strains of FAW collected from north, central and south Florida have already developed resistance to different carbamate, organophosphate, and pyrethroid insecticides (Yu 1991, 1992). Similarly, in Argentina, application of insecticides to corn against FAW did not reduce FAW population but instead diminished the establishment of parasitoids that can help to reduce the pest population (Berta et al. 2000). Moreover, reports from previous studies indicated that there is variability between the two host strains of FAW in terms of susceptibility to insecticides.

According to Adamczyk et al. (1997) and Pashley et al. (1987b), rice-strain larvae were more susceptible than the corn-strain to several insecticides, including

diazinon and carbaryl, while the reverse was true for carbofuran. Similarly, the rice strain was more susceptible than the corn-strain to transgenic Bt cotton (Adamczyk et al. 1997). Pitre (1988) reported that parathion and chlorpyrifos caused 85% mortality on third instars of FAW collected from Mississippi and Jamaica, and 50% mortality against FAW populations from Honduras.

Chemical control of FAW can be achieved through appropriate application of insecticides. However, the annual migration of FAW in to the United States from southern latitudes (Mitchell 1979, Sparks 1979, Hogg et al. 1982), the development of insecticide resistance, and the possible existence of host strains and possibly sibling species of the pest (Pashley et al. 1985, Pashley 1986), may necessitate the determination of the relationship of FAW immigration to insecticide control.

#### **1.4.6 Area-wide management**

Since FAW do not survive the prolonged freezing winter, most of the infestation in the United States comes from annual migration of populations in southern Florida and southern Texas (Barfield et al. 1980, Sparks, 1986). If the populations of the corn and/or rice strains can be controlled in the overwintering sites, it should be possible to substantially reduce or delay their northward migrations, preventing damage to crop hosts like corn.

The inability to survive extended periods of temperature below 10°C (Sparks 1986) and geographic limitation of the overwintering population creates opportunities for area-wide management of the pest and limiting the migrating population. However, the presence of two morphologically identical but behaviorally and physiologically different

host strains have complicated the efforts to understand and predict fall armyworm behavior in the field (Nagoshi and Meagher 2004). Despite this fact, preliminary studies suggested that at least the corn strain could be a good candidate for area wide management programs (Meagher and Nagoshi 2004). Knipling (1980) also strongly supported the possibilities of area-wide management of FAW through mass production and release of effective egg and/or larval parasitoids, use of pheromones for mass trapping or confusion of moths, application of microbial control agents under special circumstances, use of cultural practices to destroy cultivated or wild hosts, and releasing sterile or partially sterile moths that can compete with the field (wild) male population for mating. Integrated application of above techniques can help to suppress the FAW population in overwintering areas like Florida and also manage moths that migrate to the southern part of United States from neighboring countries like Mexico and the Caribbean basin. If integrated area-wide management is implemented, it would be feasible and practical to reduce the overwintering and migrating populations to such low levels that the surviving population could not increase to economic population levels in the normal areas of spread before the growing season ends (Knipling 1980).

In general the inability to enter into diapause, seasonal abundance of the pest starting late in winter and early spring followed by northward migration, economically justifiable losses from its damage, and more restricted overwintering areas compared to other similar pests, makes FAW amenable for area-wide management. Hence, by exploring and using the weakest link in the life cycle of the FAW, it could be possible to reduce the FAW problem.

### **1.5 Sources of genetic variation in an insect population**

Generally natural populations show a huge diversity in terms of both qualitative and quantitative traits. Part of this variability is caused by the environment; however, much of the variability in natural population variability is of genetic origin (Raven *et al.* 1986). Genetic variation within and among insect populations is the result of dispersion and reproduction (Hamrick and Godt 1990). Among several factors that may contribute for genetic variations, genetic drift and natural selection are the predominant factors that govern change at the molecular level (Kreitman and Akashi 1995).

Many of the destructive agricultural pests have broad geographic distributions and genetic differences are presumably due to the ability to adapt to local conditions (Krumm 2005). According to White and Walker (1997) genetic variation provides the basis for evolutionary change and is governed by natural selection through the interaction of genetic forces and changing environments in space and time. Genetic differences could arise due to change in the DNA structure resulting from crossing over, translocation, deletion or duplication (Cedergren *et al.* 1990) that leads to changes in the amount of DNA, the structure of the DNA, or the number or distribution of restriction sites (Dowling *et al.* 1996).

### **1.6 Genetic variability in fall army worm**

Studies on North America and Caribbean populations of fall armyworm indicated the existence of two morphologically identical strains that differ in host preference, physiology, behavior, and pesticide susceptibility (Lynch *et al.* 1983, Pashley 1986, 1988a, Pashley *et al.* 1987b, 1995; Prowell *et al.* 2004). Identification is largely

dependent on molecular markers (Nagoshi and Meagher 2003a). These two host strains can be genetically distinguished by polymorphisms in the mitochondrial cytochrome oxidase I gene (Pashley 1989), nuclear restriction fragment length polymorphisms (RFLPs) (Lu et al. 1992), variations in allozymes (Pashley 1986), amplified fragment length polymorphisms (McMichael and Prowell 1999), and a tandem-repeat genetic element called *FR* that is found in large sex-linked clusters primarily in the rice strain (Nagoshi et al 2008, Lu and Adang 1996). Nagoshi et al. (2006) also identified strain specific RFLP fragments of mtDNA for FAW populations collected from Florida, Texas, Mississippi, Georgia, and North Carolina, with an *AciI* and *SacI* site specific to the rice strain and a *BsmI* and *HinfI* sites specific to the corn strain. Levy et al. (2002) also identified *MspI* recognition site restriction fragment length polymorphism (RFLP) marker that was present only in the corn strain.

Lu et al. (1994) cloned and characterized a 189 bp *FR* sequence, which is found only in the genome of the rice strain individuals and the number of copies is higher in females than in males. Similarly, dendrograms produced by amplified fragment-length polymorphism analysis revealed two assemblages that were over 90% consistent with strain assignments based on host plant (McMichael and Prowell 1999). The corn strain is associated with large grasses such as maize and sorghum, whereas the rice-strain prefers smaller grasses such as rice and turfgrass. Busato et al. (2005) also reported the existence of two biotypes of FAW in South America that differ in physiology, host preference and susceptibility to insecticides; similar to trends observed in North American host strains of FAW.

The two strains also diverge in allelic frequencies of several glycolytic enzymes and in migratory pathways (Pashley et al. 1985). The existence of the FAW strains complicates the management of this pest as demonstrated in studies where bermudagrass specifically bred for pest resistance showed differential resistance to the corn strain of the FAW (Quisenberry and Whitford 1988).

Pashley 1986 indicated the existence of genetic similarity between the North America and South America host strains suggesting substantial gene flow between populations in the Caribbean region and mainland North America. In addition to the existence of two host specific strains in FAW, Nagoshi et al. (2007) identified four haplotype subgroups of the corn strain population using polymorphic sites in the mitochondrial DNA. Lewter et al (2006) sequenced a 608-base-pair portion of the mitochondrial cytochrome oxidase I and II genes from 71 individuals of FAW collected from Florida and Arkansas and the results showed the existence of three corn and four rice strain haplotypes in the sampled populations.

Genetic analysis of the mitochondrial cytochrome oxidase (COI) gene from corn strain populations collected from Florida identified two nucleotide sites that were polymorphic within the corn strain generating four haplotype subgroups: CS-h1, CS-h2, CS-h3, and CS-h4 (Nagoshi et al. 2007a). Comparison of the cytochrome oxidase I gene within corn-strain populations collected from Texas and Florida identified significant differences in the proportions of certain haplotypes and the differences were preserved in migrating populations, providing a molecular metric by which the source of a migrant population could be identified (Nagoshi et al. 2009). Martinelli et al. (2007) have evaluated the genetic similarity and structure of the FAW populations associated with

maize and cotton crops in Brazil using AFLP and found that there is no genetic variation between the maize and cotton populations of FAW collected in Brazil. From their results, the authors suggested that no significant structuring within the *S. frugiperda* populations associated with maize and cotton crops.

The majority of molecular marker studies performed with FAW have been focused on the corn and rice host strains issue (Nagoshi and Meagher 2003a, Prowell et al. 2004). Recently, genetic variation within and between corn strain populations of *S. frugiperda* collected from Mexico, United States, Puerto Rico, Brazil, and Argentina was studied by Clark et al. (2007) at University of Nebraska-Lincoln, Insect Molecular Genetics Laboratory, using AFLP. Their results indicated that the majority of the genetic variability was within populations and not between populations of the corn strain, indicating the presence of gene flow among FAW populations which suggested that *S. frugiperda* populations in the Western Hemisphere are an interbreeding population.

### **1.7 Behavioral and physiological differences between corn and rice strains of FAW**

Lopez-Edwards et al. (1999) observed difference in developmental time, survival, insecticide susceptibility, susceptibility to Bt, and mating compatibility in five populations of FAW collected from maize fields in Mexico. The authors suggested that two FAW strains may have developed reproductive isolation due to geographic isolation. In phytophagous insects, host use is governed by physiological factors associated with larval development or adult behaviors such as ovipositional preferences or both (Futuyama and Moreno 1988, Jaenike 1990).



Differences in host specificity between the corn and rice strains of FAW could be due to host adaptation. For example, rice-strain larvae feeding on corn displayed a slower rate of weight gain, longer developmental time, lower pupal weight, and reduced survival than when reared on bermudagrass (Pashley 1988a). Whitford et al. (1988) also observed that corn strain developed equally well on corn, bermudagrass and sorghum having significantly heavier larvae and pupae than rice strain feeding on the same hosts which developed best only on bermudagrass compared to the other hosts. The effect on larval growth rate could be due to the fact that the rice strain development is more sensitive to the type of host plant (Pashley 1988a), which may be attributed to lower inherent oxidase activity than the corn strain (Veenstra et al. 1995). In contrast, the same set of studies showed that rearing corn-strain larvae on rice or bermudagrass had no consistent negative effect on larval development or fitness (Nagoshi and Meagher 2004).

The poor performance of the rice strain on corn could be due to low consumption rate and low efficiency of converting digested food into biomass when feeding on corn compared to the corn strain because of less mixed-function oxidase activities in the rice strain (Veenstra et al. 1995). These studies suggest that larval host had a greater impact on development of rice strain than the corn strain, indicating that physiology could have facilitated specialization in one strain but not the other (Pashley 1988b). These genetic and behavioral differences between the two host strains of FAW were believed to hinder interstrain mating. However, examination of strain-specific esterase allozymes and mtDNA polymorphisms suggested the occurrence of interstrain hybridization in wild populations (Prowell 1998). On the other hand, Pashley and Martin (1987) suggested that this interstrain mating may be limited in nature. For example, when

corn-strain females were mated to rice strain males no progeny were produced and also there was no spermatophore transfer. In contrast, when rice strain females were mated to corn strain males they showed the same fertility as intrastrain matings (Pashley and Martin 1987). However, the hybrid daughters produced from the rice strain females and corn strain males failed to mate with males from either strain but were able to mate with their hybrid brothers with reduced fertility. These hybrid males were able to fertilize females of either strain although fertility was somewhat reduced compared to intrastrain matings. These results suggest significant strain-specific mate selection, such that corn strain females have a strong preference to males of the same strain or to hybrids, while rice strain females are less selective. When virgin females of each strain were used to attract males from the wild population, 60-75 % of males of both strains prefer females of the same strain, suggesting that pheromone differences might have a role in mate choice (Pashley et al. 1992, Pashley 1993).

The strongest barrier to interstrain mating is temporal partitioning of mating activities throughout the night in which the corn strain mated exclusively in the first two-thirds of the night while the rice strain mated in the last one-third of the night. Significant difference in sex pheromone composition is also observed between the two host strains which may limit interstrain mating (Groot et al. 2008). Nagoshi and Meagher (2003b) crossed the rice and corn strain and used FR sequence and a strain-specific mitochondrial marker to examine the distribution of different marker combinations in field isolated specimens and found that a large proportion of the wild population was found to carry a hybrid marker combination consistent with the frequent mating of rice strain females to corn strain males. However, the marker that is indicative of the reciprocal cross was not

found, suggesting that interstrain mating in FAW host strains is unidirectional (Nagoshi and Meagher 2003b, Ngoshi et al. 2006).

The two host strains of FAW also show differences in seasonal abundance where corn strain achieves highest larval densities in spring and midsummer whereas the rice strain is most abundant in late summer and fall (Pashley et al. 1992). Similarly Nagoshi and Meagher (2004b) observed strain specific seasonal population patterns in which the rice strain showed an increase in capture rates during the fall compared to the corn strain, despite the presence of sweet corn which is a preferred host of the corn strain. Moreover, the rice strain had shown a bimodal distribution both on turfgrass and agricultural areas with peaks in the spring and fall (Nagoshi and Meagher 2004b). These differences in population dynamics of the two host strains could indicate difference in response to seasonal environmental cues.

In general the two host strains showed genetic differences in 5 allozyme loci (Pashley 1986), mitochondrial DNA (mtDNA) haplotypes (Pashley 1989, nuclear DNA RFLP (Lu et al. 1992), mtDNA PCR-RFLP (Levy et al. 2002), and repeated DNA sequences (Lu et al. 1994), developmental difference on different host plants and insecticide susceptibility.

### **1.8 Molecular markers used in detection of genetic variability**

Molecular markers, particularly DNA based markers, have been widely applied in such areas as gene mapping and tagging, characterization of sex, genetic diversity or genetic relatedness (Kliebenstein et al. 2001, Godt and Hamrick 1998, Vos and Kuiper 1998). Molecular markers have the advantage over morphological markers in providing a

larger number of genetic-based differences and are less affected by environmental variation (Avisé 2004). The introduction of polymerase chain reaction (PCR), which is based on amplification of specific segments of DNA by one million-fold or more times using *Taq* DNA Polymerase, has revolutionized the process and application of molecular markers and helped to save time and labor (Saiki et al. 1985). Protein based markers (allozymes) were the first markers developed and used in population genetics (Vos and Kuiper 1998). However, DNA based markers are the current choice to be used in genetic studies as the DNA based markers can give efficient comparison due to the fact that genetic differences are detectable at all developmental stages of the organism unlike allozymes that may show age dependent variation of expression (Widén et al. 1994). Tools available for studying DNA structure are now able to differentiate between and among populations of organisms mainly resulted from genetic drift and natural selection (Kreitman and Akashi 1995).

The term marker refers to a locus marker, a particular place along the chromosome where each gene is located. Hence, molecular markers are all loci markers related to the DNA (Vos and Kuiper 1998). In a population genetics study, a good molecular marker is the one which is transmitted from one generation to another, polymorphic, codominant (to allow differentiation between homo and heterozygotes), neutral (all alleles have the same fitness), independent of environment, evenly distributed throughout the genome, and highly reproducible (Vos and Kuiper 1998). The commonly used molecular markers include AFLP, RAPD, RFLP, microsatellites, and allozymes. AFLP markers fulfill all characteristics of good molecular markers except being codominant. Despite their dominant nature, because of the high amount of polymorphism

they can detect and other several advantages, AFLPs are very commonly used molecular markers.

### **1.8.1 Restriction fragment length polymorphism (RFLP)**

RFLP markers detect differences within a species in the length of DNA fragments generated by specific restriction endonucleases. Restriction enzyme analyses are versatile, providing information on the nature, as well as the extent, of differences between sequences in nuclear or mitochondrial DNA (Dowling et al. 1996). RFLP variations are caused by mutations that create or eliminate recognition sites for the restriction enzymes which generate the restriction fragments (Hoy 2003, Smith 2005). RFLP analysis can be used to analyze clonal populations, heterozygosity, relatedness, geographic variation, hybridization, species boundaries, and phylogenies. RFLP markers are used to analyze mtDNA, single copy nuclear DNA, and repeated sequences with ribosomal DNA (Hoy 2003). The restriction fragments of the target DNA are visualized in the gel by several methods, including staining with ethidium bromide or probing Southern blots with labeled probes.

The DNA digested by endonucleases is size-separated by electrophoresis in an agarose or polyacrylamide gel. DNA fragments of known length (size standards) are run on each gel to serve as an internal standard and to allow the size of the experimental fragments to be estimated. The digested DNA is then transferred to a nylon membrane via Southern blotting and the membrane is probed with a radioactive or fluorescent labeled probe of known sequence. However, Southern blots require a suitable probe with sufficient sequence similarity to the target DNA so that a stable hybrid can be formed at

moderate to high stringency. The membrane bound DNA is heat denatured and some probe sequences bind to complementary sites in the DNA restriction digest. The membrane is exposed to x-ray film, which is photographically developed into an autoradiogram (Smith 2005).

Some of the disadvantages of traditional RFLP are eliminated by PCR-RFLP for analyzing population variation using DNA isolated from individual organisms (Karl and Avise 1993). Since PCR-RFLP is able to amplify fragments of DNA of interest, there is no need for large amounts of DNA and radio-labeled probes. In PCR-RFLP, nuclear DNA is amplified by the PCR using primers and digested with restriction enzymes. The PCR product is visualized after electrophoresis by staining with ethidium bromide.

The advantage of PCR-RFLP over the traditional RFLP is that DNA extracted from a single individual is sufficient, after amplification, to provide electrophoretic bands that can be visualized without having to be hybridized with radio labeled probes. Levy et al. (2002) used PCR-RFLP to amplify a 569 base pairs region of the mitochondrial gene COI of the corn and rice strains and the target RLFP marker was found in corn strain but not in the rice strain of FAW. Nagoshi et al. (2006) also amplified a 568 bp region of the COI gene using PCR-RFLP and identified four RFLP markers that are specific to the host strains. Clark (2000) successfully used RFLP in identification and parsimony analysis into species clades of *Diabrotica*. Meagher and Meagher (2003) used RFLP and PCR-RFLP to identify strains of FAW from moths collected in Florida using mitochondrial DNA.

Although PCR-RFLP has solved the problems of traditional RFLP, only limited loci are analyzed limiting the possibility of detecting genetic variation and specific

primers are needed to successfully amplify a given region of interest. This limits application of PCR-RFLP in unknown organisms or source of DNA, which is not a problem in other molecular markers like AFLP.

### **1.8.2 Random Amplification of Polymorphic DNA (RAPD)-PCR**

RAPD markers result from differences in primer binding sites that allow the visualization of polymorphisms within a species. Amplified DNA fragments can be detected as bands in ethidium bromide stained agarose gels through electrophoresis. RAPD-PCR bands are considered as dominant loci in diploid organisms, and scored as present or absent. PCR amplifications of genomic DNA with RAPD primers produce numerous fragments that can be exceedingly variable among individuals and have proven useful for parentage analysis (Hadrys et al. 1992). RAPDs commonly reveal genetic polymorphisms and can provide molecular markers over a range of taxonomic scales, especially in species that were previously inaccessible to study at the molecular level. According to Hoy (2003) RAPD-PCR loci can be used to determine paternity, kinship, and hybridization, as well as to estimate population heterozygosity, effective population size, identify biotypes and cryptic species, and measure genetic distance between populations and interpopulation diversity.

RAPD-PCR has been also used to develop genetic maps and identify molecular markers in populations or species, as well as determine paternity in dragonflies (Hadrys et al. 1992). Maretinelli et al. (2006) used RAPD-PCR to investigate the genetic variability among 10 populations of *S. frugiperda* collected from maize and cotton crops in Brazil. Wilkerson et al. (1993) have separated two morphologically identical

mosquitoes, *Anopheles gambiae* (Giles) and *A. arabiensis* (G.), by using the RAPD-PCR technique. RAPD markers have been also used to study genetic variation in European corn borer (Pornkulwat 1998, Saldanha 2000). This technique has been widely used for species identification and linkage maps because of its simplicity and rapidity with small amounts of DNA required or not requiring any previous DNA sequence information. The other advantage of RAPD-PCR is that it is useful to identify hundreds of new markers in a short time, which allows genetic maps to be developed rapidly; particularly it is valuable for genome mapping in those species for which other genetic markers are lacking or rare (Laurent et al. 1998). RAPD markers have been also used for discriminating between morphologically identical organisms such as *C. hominivorax* and *C. macellaria* (Fabricius) (Skoda et al. 2002) and distinguishing geographical populations of the velvetbean caterpillar, *Anticarsia gemmatilis* Hübner (Sosa-Gómez (2004).

The major disadvantage of RAPD-PCR is its lack of reproducibility in which different RAPD banding patterns can be obtained if different DNA extraction methods or polymerases are used (Micheli et al. 1994). Moreover, RAPD-PCR is highly sensitive to both DNA template concentration and quality, so that DNA bands may vary in intensity or even disappear if template concentration is not controlled or DNA is sheared (Khandka et al. 1997). It is also difficult to reproduce RAPD-PCR results if different PCR machines or pipettors are used, resulting in different temperature cycling conditions or different concentrations of the PCR mixture (He et al. 1994, Schweder et al. 1995). Hence, use of RAPD markers to calculate genetic similarity coefficients can result in false positives and false negatives if RAPD artifacts are present (Lamboy 1994).



### **1.8.3 Microsatellites**

Microsatellite PCR primers, also called simple sequence repeats (SSRs), are tandem arrays of 2 to 5 base repeat units that are widely distributed in eukaryotic DNA. In arthropods, the SSRs repeats most often consist of repeats of dinucleotides (AC,AT, AG), trinucleotides (AGC, AAC,AAT) or tetranucleotides (ACAT,AAAT, AAAC) (Toth et al. 2000). Microsatellites typically are scattered throughout the chromosomes of most organisms and found both in protein-coding and noncoding regions. The rate of mutation in microsatellites is very high and they are thought to play a significant role in genome evolution by creating and maintaining quantitative genetic variation (Kashi et al. 1997).

The high rate of mutation in microsatellites is due to DNA polymerase slippage (strand dissociation and reassociation) and unequal recombination. Several advantages have contributed to the success of microsatellite markers. These include their ability to detect single loci and their specific chromosome localization (Mith 2005). Microsatellite markers can identify individual arthropods or their progeny, evaluate kinship, mating, and reveal differentiation among closely related populations in the field (Burke 1989). Moreover, microsatellites are useful in monitoring establishment and dispersal of specific biotypes in an insect population. However, microsatellites are relatively time consuming and expensive to develop as microsatellite sequences differ in different organisms, even in closely related species so that microsatellite sequence data must be obtained for each species under study (Neve and Meglecz 2000).

#### **1.8.4 Allozymes**

Allozymes are codominant protein variants (markers) that can be visualized by appropriate staining and agarose gel electrophoresis. They have been used to analyze mating systems, inbreeding, genetic drift, hybridization, effective population size, degree of genetic differentiation among populations, and migration (Hoy 2003). Allozymes are also useful in determining genetic relationships among species through assessment of affiliations of rare taxa and predict relative endangerment among species (Miller and Westfall 2010).

Protein electrophoresis is one of the most cost effective techniques and is relatively easy to perform (Hoy 2003). However, allozyme markers represent genes that are expressed in the organism at the time of sampling, yielding different information about genetic variation within species than revealed by other molecular markers. This may underestimate the detection of genetic variation by 30% of the actual genetic diversity as determined by DNA-based methods (Hoy 2003, Miller and Westfall 2010). Moreover, protein electrophoresis may not detect sufficient variation to answer some questions, and the number of analyses that can be performed with very small insects may be limited because of inadequate amounts of proteins. Proteins are also less stable than DNA and thus may be more sensitive to handling and storage problems. Therefore, when estimates of total genetic variation are important, allozymes are best used in conjunction with other markers.

### **1.8.5 Amplified fragment length polymorphism (AFLP)**

AFLP is a powerful DNA ‘finger printing’ technology applicable to any organism or complexity such as prokaryotes, plants, animals, and human without the need for prior knowledge of the sequence (Vuylsteke et al. 2007). The AFLP technique was originally developed for the construction of high density linkage maps for application in positional cloning of genes and molecular breeding (Vuylsteke et al. 2007). The AFLP approach is based on selective PCR amplification of a subset of genomic restriction fragments. The technique combines the reliability of RFLP’s and power of PCR in that AFLP fingerprints are actually restriction fragment length polymorphisms (RFLP’s) visualized after selective PCR amplification of subsets of restriction fragments of genomic DNA (Vos et al. 1995). However, unlike RFLP, the AFLP technique results in the presence or absence of fragments rather than difference in the length of fragments in RFLP.

In AFLP, ‘finger prints’ are produced without prior sequence knowledge using a limited set of generic primers and it is a powerful tool in detecting polymorphisms originating from mutations that can create or delete restriction sites, inversions or deletions between two restriction sites. AFLP ‘finger prints’ allow to survey numerous independent loci per run and are thus well suited for identification of markers linked to specific phenotypes. Due to complexity of the AFLP procedure, it needs calibrations for any specific system. Many parameters like number and type of enzymes used, type, number, and combination of primers and the PCR applications conditions can be manipulated to fit the specific AFLP ‘finger print’ required.

The most commonly used restriction enzyme combinations in AFLP are EcoRI (a rare cutter having 6 bp recognition sites) and MseI (a frequent cutter having 4 bp

recognition sites). Selection of appropriate primers is very important in determining the level of amplification. Primers used in AFLP have three parts: the 5' part corresponding to the adapter, the restriction site sequence and the 3' selective nucleotides.

The number of restriction fragments amplified in a single AFLP reaction is determined by two major factors: the number of selective nucleotides and their C and G composition (Grandillo and Fulton 2002). This intern will determine the selectivity of the reaction; the more Cs and Gs used as selective nucleotides in the amplification primers, the fewer the number of fragments that will be amplified. AFLP is seen as a valuable genetic marker system in population genetics, ecology and evolution as it brings key answers to major biological issues in a wide variety of organisms, like fungi, birds, fish, humans, and plants (Kis-Papo et al. 2003, Irwin et al. 2005, Barluenga et al. 2006, Prochazka et al. 2001).

Although AFLP is a powerful genetic marker in population genetic analysis, it has certain limitations. First, polymorphic loci are scored for two alleles i.e. presence or absence of the band. The other limitation is that AFLP is a dominant marker and does not distinguish homozygotes from heterozygotes unless pedigree analysis is done (Bonin et al. 2007). However, this shortcoming of AFLP in the lack of information resulting from dominance is counterbalanced by the high numbers of polymorphic bands that AFLP produces (Gerber et al. 2000). There are two methods for extracting AFLP data, band based approach (presence or absence of bands), and allele frequency based approach which is based on estimation of allele frequencies at each locus (Bonin et al. 2007). Estimation of allele frequency data from dominant biallelic data in diploids is based on either Hardy-Weinberg hypothesis or on the known inbreeding coefficient.

Like any other experiments, AFLP needs an experimental design that includes a sampling strategy which helps to determine the number of bands and individuals to sample. Sample size is influenced by various parameters including mating system, effective population size, and existing level of population structure which can influence the accuracy of population genetics estimates (Mohammadi and Prasanna 2003, Mendelson and Shaw 2005). Because AFLP employs a dominant biallelic data, it needs genotyping of 2-10 times more individuals per population than codominant markers like microsatellites to achieve the same level of accuracy. For classical surveys of genetic diversity, population structure, and genetic relatedness, there is usually a range in the number of markers below which sampling variance is too high and estimates are thus not reliable. However, sampling above the range also does not necessarily increase the power (Hollingsworth and Ennos 2004). Generally, genotyping of 30 individuals and scoring of 200 AFLP markers per individual sample can yield accurate results in population genetics studies (Krauss 2000, Bonin et al. 2007).

Even though AFLP is highly reproducible, care should be taken to minimize genotyping errors, mainly allele homoplasy and scoring errors. Allele homoplasy refers to migration of nonhomologous fragments at the same position in an electrophoretic profile, or the loss of the same fragment by different mutations (Meudt and Clarke 2007, Simmons et al. 2007). This can decrease the estimates of genetic diversity and can limit the power of the analysis (Koopman and Gort 2004, Meudt and Clarke 2007). In order to reduce the problem of allele homoplasy, while choosing AFLP markers one has to confine the analyses to the intraspecific level and avoid transfer of markers between species, favor primer combinations that can generate clearly readable and exploitable

profiles (bands), give preference to longer bands as markers, and assess the extent of fragment homoplasy by means of sequencing (Rombauts 2003). The majority of errors occurred in genotyping AFLP data sets are scoring errors (Bonin et al. 2004) which arise from difficulty and subjectivity in reading profiles correctly, especially when there are differences in band intensity between individuals or between runs. Double readings of profiles and running replicates can help to minimize scoring errors (Bonin et al. 2004).

Alamalakala (2002) revealed that AFLP can consistently distinguish between European and North American populations of *O. nubilalis*. Krumm et al. (2008) used AFLP to measure genetic variation within and between sub-populations to infer genetic diversity and gene flow for *O. nubilalis*. Martinelli et al. (2007) also used AFLP to evaluate the genetic similarity and structure of the FAW populations associated with maize and cotton crops in Brazil. Clark et al. (2007) also used AFLP to determine genetic variability of FAW populations in the Western Hemisphere.

### **1.9 Applications and principles of AFLP**

AFLP is a new molecular fingerprinting technique that can be applied to DNAs of any source or complexity (Bleas et al. 1998). The potential applications of AFLP includes monitoring inheritance of agronomic traits in plant and animal breeding, diagnostics of genetically inherited diseases, pedigree analysis, forensic typing, parentage analysis, screening of DNA markers linked to genetic traits, microbial typing, genetic diversity or relatedness and ecological and phylogenetic studies (Bleas et al. 1998, Vos and Kuiper 1998). The major advantage of AFLP over the other DNA-based molecular markers is its capacity to inspect an entire genome for polymorphism and its

reproducibility. The nuclear origin of AFLP markers is attractive because markers derived from uniparentally inherited organellar genomes (chloroplast and mitochondrial genomes) might not be sufficiently variable, or even appropriate particularly in organisms where processes such as hybridization are important (Meudt and Clarke 2007).

AFLP principle is based on selective amplification of restriction fragments from a restriction digest of total genomic DNA using PCR in which molecular genetic polymorphisms are identified by the presence or absence of DNA fragments. The AFLP technique is developed by Zabeau and Vos (1993) and is patented by Keygene NV (Wageningen, The Netherlands) (Bleas et al. 1998). The major steps in AFLP include digestion of genomic DNA, ligation of adapters, amplification, and gel analysis. Total genomic DNA is digested using two restriction enzymes i.e. a rare cutting enzyme with 6 to 8 base recognition (e.g. *EcoRI*, *AseI*, *HindIII*, *ApaI* and *PstI*), in combination with a frequent cutting enzyme of 4-base recognition (e.g. *MseI* and *TaqI*). The high degree of specificity of these restriction enzymes enables the production of reproducible DNA fragments, and the use of different enzymes allows the researcher to manipulate the number and complexity of fragments to be produced (Bleas et al. 1998).

The type of fragments produced in AFLP could be fragments cut by individual restriction enzymes or by both. The frequent cutter helps to generate numerous small DNA fragments, which can be amplified and separated on sequence gels. On the other hand, the number of fragments to be amplified is reduced by using the rare cutter since only the rare cutter/frequent cutter fragments are amplified. Moreover, the use of two restriction enzymes makes it possible to label one strand of the PCR product to be visualized during electrophoresis. Once restriction fragments are produced, 10-30 bp long

double-stranded nucleotide adapters are ligated to the DNA fragments using T4 DNA ligase to serve as primer binding sites for PCR amplification. The adapters have a core sequence and an enzyme specific sequence that allow the ligation of the adapters at EcoRI or MseI restriction sites of the fragments. Ligation of adapters to the restricted sites of the fragments alters the restriction site thereby preventing a second restriction from taking place after ligation has occurred.

Once ligation of adapters is completed, preamplification is done using preamplification primers mix that allows the first selection of fragments by amplifying only the DNA fragments that have ligated an adapter at both ends. The PCR process in the preamplification stage involves repetitive series of cycles that include template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase resulting in an exponential accumulation of specific fragments whose termini are defined by the 5' ends of the primers (Erlich et al. 1991).

Following preamplification, selective amplification is done in order to restrict the level of polymorphism, limit the number of fragments to be amplified, and label the DNA with florescent dye which allows the visualization of the DNA during migration in the gel. Most commonly the primer corresponding to the rare cutting restriction enzyme is labeled by phosphorylating the 5'-end (Bleas et al. 1998).

In the selective amplification, three more nucleotides are added at the 3' end of the primer sequence used in the preamplification, hence, only restriction fragments in which the nucleotides flanking the restriction site matching the selective nucleotides will be amplified. AFLP marker polymorphism is identified by the presence or absence of



DNA fragments following analysis on polyacrylamide gel which is visualized by autoradiography.

In the AFLP technique, the amplification is not completely random nor is the primer based on a known host DNA sequence. This allows for DNA fingerprinting for DNAs of any origin or complexity (Vos et al. 1995). Hence, AFLP provides the advantage of random amplification and repeatability to study genetic variability of insect populations including FAW.

### **1.10 Justification of the study**

The FAW is one of the most economically important pests of various crops and grasses in the Western Hemisphere. Understanding genetic diversity and gene flow of a target pest species is necessary prior to large scale efforts aimed at control of insect pests (Martinelli et al. 2007). Previous studies on the genetic diversity of the pest indicated the existence of two morphologically identical host specific strains of the FAW, one associated mainly with large grasses like maize and sorghum (the corn strain) and the other associated with rice, Bermuda grass and other small grasses (the rice strain) (Pashley 1986, 1988a; Pashley et al. 1987a, 1995). It is also reported that the two strains show variability in their physiology, mating and migration behavior, and pesticide susceptibility (Lynch et al. 1983, Pashley et al. 1985, 1987b, 1995; Pashley 1986, 1988a; Prowell et al. 2004).

Understanding within and between population genetic diversity of FAW could play an important role to develop appropriate management strategies including area wide management and insecticide resistance management programs to minimize crop losses.

Attempts have been made by different researchers to study the genetic diversity of within and between populations of the FAW using AFLP, RFLP and allozyme molecular markers (Lu et al. 1992, McMichael and Prowell 1999, Clark et al. 2007, Martinelli et al. 2007).

Analysis of mitochondrial COI gene in FAW corn strains collected from Brazil and Florida showed that the Brazil corn-strain population is different from corn strain FAW found in Florida (Nagoshi et al. 2007). Further sequence analysis of COI locus revealed two highly polymorphic sites in the corn strain population that produced four different haplotype subgroups which did not show seasonal or host specificities. However, in most of the studies FAW samples were collected from limited geographic areas and only few individuals per sample and few loci per individual were studied. Further geographic genetic analysis of the FWA with a large sample size and loci could reveal more comprehensive genetic structure of the pest (corn strain) which is crucial for designing and implementation of pest management programs.

A good understanding of genetic diversity within and between populations can be used to determine the rate of spread of insecticide resistance, which would assist in the preservation of successful insecticide use as well as deployment of transgenics. For example, we have already observed that FAW population in Puerto Rico has developed resistance to Bt corn varieties (personal observation) and there is a situation that insecticides are sprayed 3 time a week on the same crop and up to 25 sprays have been made in a single crop cycle to control FAW. Hence, there is a need to know the genetic diversity of this economically important pest by using large sample size and molecular markers. Moreover, in Puerto Rico where most of the seed companies grow their corn

nurseries throughout the year and insecticide application is the major control option, insecticide resistance monitoring is important.

## **1.11 Research Objectives and Hypotheses**

### **1.11.1 Objectives**

1. Study the genetic variability and gene flow of FAW in the Western Hemisphere.
2. Test susceptibility of Puerto Rico FAW population to insecticides being used to control the pest.

### **1.11.2 Hypotheses**

1. There is no geographic genetic variability in FAW populations in the Western Hemisphere.
2. High FAW pressure and dependency on insecticides to control the pest has led to development of insecticide resistant FAW population in Puerto Rico.

**CHAPTER 2**

**GENETIC VARIABILITY AND GENE FLOW OF FALL**

**ARMYWORM, *SPODOPTERA FRUGIPERDA* (J. E. SMITH), IN THE**

**WESTERN HEMISPHERE**

## **2. GENETIC VARIABILITY AND GENE FLOW OF FALL ARMYWORM, *SPODOPTERA FRUGIPERDA* (J. E. SMITH), IN THE WESTERN HEMISPHERE**

### **2.1 Abstract**

The fall armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is a migratory and polyphagous pest of both cultivated and uncultivated plant species in the Western Hemisphere extending from the United States through Central America to South America and in the Caribbean. It causes substantial losses in economically important crops like maize, sorghum, forage grasses, turf grasses, rice, cotton, peanuts and others. The species passes through 6 larval instars during its life cycle and the total life cycle can be completed in 30-90 days depending on temperature and other factors.

Previous studies on genetic diversity of FAW indicated the existence of two morphologically identical strains that differ in host preference, physiology, behavior, and pesticide susceptibility. One strain specialized in feeding on corn, sorghum and other large grasses and is named the corn strain. The other, called the rice strain, mainly feeds on rice, Bermuda grass, and other small grass species. Identification of the two host strains is mainly based on use of different molecular markers. Most of previous studies on genetic variation of FAW have focused on identification of these two host strains; there is limited information on the geographic genetic variation which is important to design appropriate pest management options. To bridge this information gap, in the present study we have investigated the geographic genetic diversity of FAW (corn strain) by collecting representative samples from the Western Hemisphere i.e. United States,

Argentina, Panama, and Puerto Rico, and analyzing large sample size and molecular markers using AFLP.

The results showed that the 221 AFLP markers analyzed in the present study are sufficient and explained more than 95.6% of the variability observed in the FAW populations. Analysis of molecular variance (AMOVA) revealed that the majority (71.2%) of the total variation is within populations; the remaining 28% of the variation was among populations within a group indicating the presence of significant gene flow among FAW populations in the Western Hemisphere. Similarly, dendrograms of results from cluster analysis showed the lack of genetic structuring i.e. the samples were randomly clustered in close proximity.

The isolation by distance analysis indicated that there is no significant correlation between genetic dissimilarity and geographic distance for the entire population, suggesting the presence of substantial gene flow. However, the dependency of genetic distance on geographic distance was significant for FAW samples collected from Argentina; probably due to the presence of local barriers that led to change in allele frequency and subsequent genetic isolation. The results of the study are important to develop management strategies and resistance monitoring.

## **2.2 Introduction**

The fall armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is a native pest to the subtropical and tropical regions of the Western Hemisphere from the United States through Central America to South America and in the Caribbean (Knipling 1980, Pashley et al. 1985, Pashley 1986, 1988b). FAW do not survive conditions of prolonged freezing and its infestation in the central and northern

parts of United States through Canada during spring to fall seasons comes from annual migration of the populations that overwinters in southern Florida and Texas (Barfield et al. 1980). This migratory and polyphagous pest is capable of causing substantial losses in maize, sorghum, forage grasses, turf grasses, rice, cotton, and peanut production (Sparks 1979, Hall 1988). Despite its wide host range throughout the Western Hemisphere, the FAW primarily feeds on corn, rice, Bermuda grass and other grass species and this polyphagous behavior has been attributed to dietary generalization (Luginbill, 1928). FAW passes through 6 larval instars during its life cycle with multiple generations per year; the total life cycle can be completed in 30 days during the summer, but the duration can be extended to 60 days in the spring and autumn, and 80 to 90 days during the winter (Capinera 2001).

Studies on genetic diversity and gene flow of FAW populations from North American and Caribbean regions indicated the existence of two morphologically identical strains that differ in host preference, physiology, behavior, and pesticide susceptibility (Lynch et al. 1983, Pashley 1986, 1988a; Pashley et al. 1995, Prowell et al. 2004). One strain was identified as corn strain that mainly feeds on corn, sorghum, and other large grasses; the other strain is called the rice strain and mainly feeds on rice, Bermuda grass, and other small grasses (Pashley 1986). The two strains are morphologically identical and identification is largely dependent on molecular markers (Nagoshi and Meagher 2003a). These two host strains can be genetically distinguished by polymorphisms in the mitochondrial cytochrome oxidase I gene (Pashley 1989), nuclear restriction fragment length polymorphisms (RFLPs) (Lu et al. 1992), variations in allozymes (Pashley 1986),

amplified fragment length polymorphisms (McMichael and Prowell 1999), and tandem repeats (Lu and Adang 1996, Nagoshi et al 2008).

Recently, Clark et al. (2007) analyzed the genetic diversity of the corn strain populations from Mexico, United States, Puerto Rico, Brazil, and Argentina; the results suggested the presence of continuous gene flow between the different geographic areas where FAW was sampled. Martinelli et al. (2007) also found no significant structuring within the *S. frugiperda* populations associated with maize and cotton crops in Brazil. However, other studies have shown the presence of physiological and behavioral differences between populations of FAW that are consistent with reproductive isolation caused by geographical separation (Young 1979). Hence, it is still unclear the extent to which the dispersed populations of fall armyworm in the Western Hemisphere genetically interact.

Analysis of population genetic structure of invasive pest species may help to identify the origin, the number of introductions and the spread of the infestation of a pest in an area and aid in designing appropriate management strategies. Despite the possible benefits that population genetic analysis of the FAW may provide towards understanding dispersal, monitoring the spread of insecticide resistance, and implementation of area-wide management programs relatively little information is available in this area. Most of the FAW research has focused on identifying behavioral and physiological differences between the two strains; only few studies were done on geographic variability and these used limited samples and few molecular markers. This suggests that there is a need to study the geographic genetic variability within the corn strain of FAW; it attacks major crops like corn, cotton, and sorghum and is the most economically important pest



throughout the Western Hemisphere (Nagoshi et al 2006). Hence, the objective of the present study was to investigate the genetic diversity and gene flow of FAW by analyzing large sample size and molecular markers using AFLP.

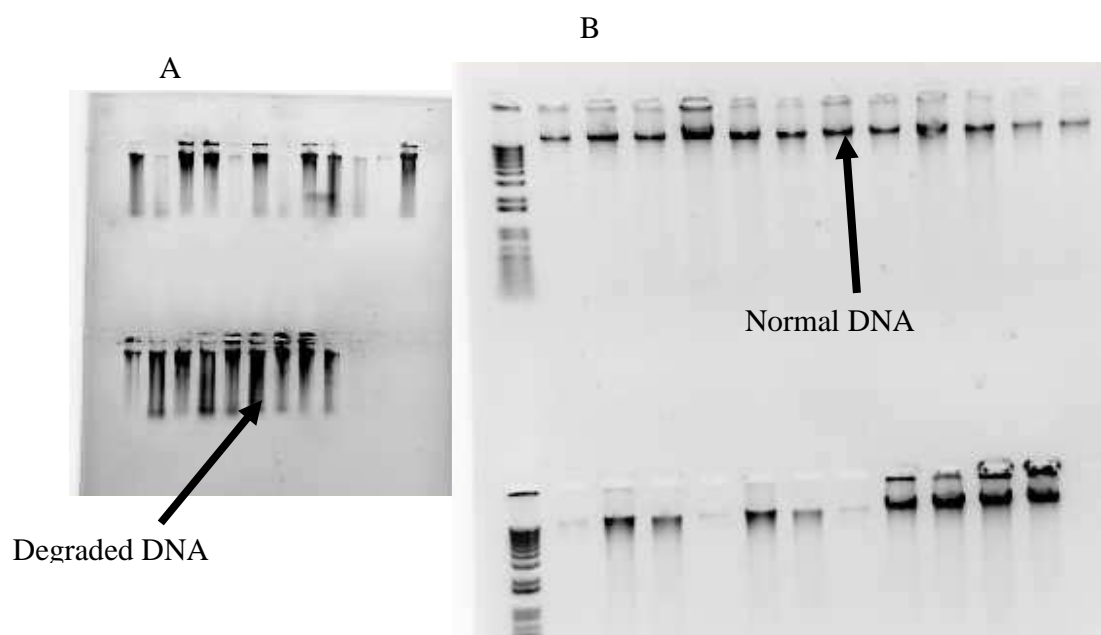
### 2.3 Materials and Methods

**Insect material collection:** For this study, FAW samples were collected from the United States, Argentina, Puerto Rico, and Panama (Fig. 3). Samples were collected from maize, sorghum, and peanut fields. Detail of collection sites and host plants is indicated in Table 1 and Appendix 1. FAW larvae collected for this study were received either live with diet, preserved in 95% ethyl alcohol (ETOH) or lyophilized. The samples that were received live on natural host plant or artificial diet were kept in a growth chamber with artificial diet until they grow to a larger size (fourth instar and above) and then they were collected in plastic boxes or vials and immediately kept in a -80°C freezer until DNA extraction. For samples collected in alcohol, the alcohol was changed 2-3 times following collection to avoid DNA degradation due to dilution of the alcohol by the water released from the larvae and then stored in a -80°C freezer. Lyophilized samples were stored dry on the laboratory bench at room temperature or were placed in the -80°C freezer until they are extracted (Clark et al. 2009). No significant difference either in the amount or quality of DNA was observed in samples preserved (collected) in the three methods. Clark et al. (2009) also did not observe significant differences in quantity or quality of DNA in FAW larvae that were preserved in 95% Ethyl alcohol, lyophilized or fresh samples. However, it should be noted that samples should be lyophilized or kept in 95% ETOH before the DNA is degraded, because some of the larvae may die during collection

and transportation to the laboratory before they are placed in ETOH or lyophilized. Samples that are degraded before DNA extraction show a continuous smear on the agarose gel (Fig 4A) instead of a clear single DNA band (Fig 4B). One of the major problems to get samples from abroad is obtaining both the import and export permits from the respective countries, and shipment of samples in alcohol due to various restrictions for security reasons.



**Figure 3.** Fall armyworm sampling regions in the Western Hemisphere.



**Figure 4.** Agarose gel picture of degraded DNA sample (4A) and normal DNA sample (4B).

The shipment (security) problem for air line shipment can be solved by lyophilization. Although importation of FAW into the United States from South American countries is allowed according to United States Department of Agriculture, Animal and Plant Health Inspection Service regulation, shipping outside from South American countries like Brazil is another problem since they have their own restriction (Clark et al. 2009). Samples collected from Brazil for this study are still in Brazil due to restrictions by Brazilian authorities on genetic resource movement out of the country.

**Table1.** List of FAW collection sites, host plants, insect life stages and date of collections.

<b>Sample # (ID)</b>	<b>Country</b>	<b>State/ County</b>	<b>Coordinates</b>	<b>Host Plant</b>	<b>Life Stage</b>	<b>Collection Date</b>
LA1	USA	Louisiana/ St. Joseph		Corn	Lar. + pupa	7.10.08
LA2	USA	Louisiana/ Winns Boro		corn	Lar. + Pupa	7.10.08
TX1	USA	Texas, Hartley	N35°45.52585 W102°23.8988	Sorghum	Larva	7.31.08
TX2	USA	Texas/ Hale county		Corn + sorghum	Larva	8.05.08
TX4	USA	Texas, Hale county		Corn + sorghum	Larva	8.08.08
NE	USA	Nebraska/ Elkhorn	N35°43.5175 W96°25.468	Corn	Larva	8.14.08
MS	USA	Mississippi/ Washington county	N33°.27197 W091°.02464	Corn	Larva	9.25.08
TX3	USA	Texas/ Lubbock		Corn	Larva	2008
AL	USA	Alabama/Macon		Cotton	Larva	9.2.09
IA	USA	Iowa/ Johnston		Corn	Larva	2009
PAN1	Panama	Pacora		Corn	Larva	6.11.09
PAN2	Panama	Chepo		corn	Larva	6.24.09
ARG1	Argentina	Alata Garacia		Corn	Larva	1.8.09
ARG2	Argentina	Canada Luque		Corn	Larva	1.9.09
ARG3	Argentina	Tadil/BS,A.S.		Corn	Larva	1.26.09
ARG4	Argentina	Camilo Alado		Corn	Larva	1.28.09
ARG5	Argentina	La Oria/BS,AS.		Corn	Larva	1.22.09
ARG6	Argentina	La Rosa/BS.AS.		Corn	Larva	1.23.09
ARG7	Argentina	Salto/BS.AS.		Corn	Larva	1.23.09

\*Lar = Larva

**Table 1.** Continued-FAW collection sites...

<b>Sample # (ID)</b>	<b>Country</b>	<b>State/County</b>	<b>Coordinates</b>	<b>Host Plant</b>	<b>Life Stage</b>	<b>Collection Date</b>
ARG8	Argentina	Charata, Chaco		Corn	Larva	1.28.09
ARG9	Argentina	Fraga/Santa Fe		Corn	Larva	1.27.09
ARG10	Argentina	Fraga/Santa Fe		Corn	Larva	1.27.09
ARG11	Argentina	Guerrico/BS.AS		Corn	Larva	1.27.09
ARG12	Argentina	Tanajera/Sgo. Del Estero		Corn	Larva	2.9.09
ARG13	Argentina	Sanda/Tucuma'n		Corn	Larva	2.13.09
ARG14	Argentina	El Azul/Burrugacu		Corn	Larva	2.13.09
ARG15-	Argentina	Ruta/ SanLuis		Corn	Larva	2.4.09
ARG16	Argentina	Ruta/ SanLuis		Corn	Larva	2.4.09
ARG17	Argentina	Rio/Cordoba		Corn	Larva	2.5.09
PR1	USA	Puerto Rico/ Santa Isabel		Corn	Larva	5.17.10
PR2	USA	Puerto Rico/ Isabella		Corn	Larva	5.13.10

**DNA extraction and quantification:** DNA was extracted from whole larvae/pupae of FAW following the CTAB method originally developed by Black and Duteau (1997) and modified by Clark (2005). Before starting DNA extraction, the lyophilized or alcohol preserved larvae were washed and soaked in double distilled autoclaved water for 10 minutes. This enabled the larvae to be re-hydrated and the tissue becomes soft for removing the gut. Once the larva is softened, the gut was removed by

making a small incision with scalpel and tightly holding at the head with forceps. Similar washing and dissection procedure was used for samples received at pupal stage.

The scalpels and forceps used in dissecting samples were washed with 95% ethyl alcohol and cleaned with Kimwipes between individual samples and new wax paper was used for each sample during dissection. For larger size larvae I used only the thoracic region, because from preliminary DNA extraction experiments I have observed that using excessively large tissue decreases the quality of the DNA unless the amount of extraction buffer is increased proportionally which may need larger extraction tubes and further optimization of the protocol. Then, larval/pupal tissue was placed in 1.5 ml autoclaved Eppendorf tubes and homogenized manually with a pestle in 250  $\mu$ l 1 Hexadecyltrimethylammoniumbromide (CTAB), a cationic extraction buffer (100mM Tris-HCL pH 8.0, 1.4m NaCl, 0.02m EDTA, 2% CTAB, and 0.2% beta-mercaptoethanol). Additional 250  $\mu$ l of CTAB buffer, and 10  $\mu$ l proteinase K (concentration of 200  $\mu$ g/ml), was added to the homogenate and vortexed at low speed and then incubated for 1 h at 65°C. The main function of the CTAB is to solubilise cellular membranes, denature proteins and form complex with DNA, and the proteinase K is used to digest proteins found in the insect tissue. RNA was removed from the homogenate by adding 15  $\mu$ l RNase A (500  $\mu$ g/ml concentration) to the homogenate in each tube and incubating for additional 2 h at 37°C.

Applying RNase too soon after removing from the 65°C heating block or adding while the sample is still on the 65°C will degrade the RNase and result in simultaneous extraction of RNA with the DNA (Fig 5). After RNA and proteins are removed from the samples, the homogenate was centrifuged at 14,000 revolutions per minute (rpm) for 5

min at room temperature so that the tissue will settle at the bottom of the tube. The supernatant was removed with a 1000  $\mu$ l pipette leaving behind as many debris as possible and then the CTAB-DNA complex was separated from the remaining cellular debris with 500  $\mu$ l of chloroform: isoamyl alcohol (24: 1) by centrifugation at 14,000 rpm for 20 min.

After the first chloroform extraction, 3 phases are observed in the tube i.e. the upper clear aquatic phase containing DNA, the middle thin layer containing the debris and the lower denser phase containing secondary compounds like proteins and carbohydrates. Then, the upper aqueous phase was transferred into a new autoclaved Eppendorf tube and the chloroform: isoamyl step was repeated and the supernatant was collected into a new Eppendorf tube. The DNA was precipitated by adding 400  $\mu$ l chilled ( $-20^{\circ}\text{C}$ ) isopropanol to the supernatant and incubated at  $4^{\circ}\text{C}$  for 8 h or left overnight. After incubation, the precipitate was centrifuged at 12,000 rpm and  $4^{\circ}\text{C}$  for 30 min. At this stage the DNA pellet was visible at the base of the tubes. The isopropanol was carefully decanted off and the DNA pellet was retained and rinsed with 500  $\mu$ l 100% chilled ETOH and centrifuged at 12,000 rpm at  $4^{\circ}\text{C}$  for 5 min to remove the salts present in the extraction reagents. Again, the supernatant (alcohol) was poured off and the pellet was rinsed with 500  $\mu$ l of 70% cold ETOH and centrifuged for 5 min.



**Figure 5.** Simultaneous extraction of RNA with DNA.

Then, the ETOH was again decanted off and the pellet was air dried at room temperature for 30-45 min. Once all the alcohol has evaporated, fifty ml 1xTE buffer (10mM Tris-HCL pH 8.0; 0.1mM EDTA) was added to the DNA pellet and stored at 4°C for a short time ( 1-2 weeks) and transferred to -20°C. Prior to starting the AFLP steps, DNA from individual samples was quantified using both 1% agarose gel electrophoresis at 60 volts (with a known standard) for 20-30 min and Nanodrop® spectrophotometer (ND-1000 V3.5.1) (Thermo Scientific, Wilmington, DE).

It is possible to get information both on quantity and quality of the DNA from the Nanodrop® spectrophotometer reading alone but the 280/260 nm ratio that indicates that quality of the DNA did not tell you whether the DNA is degraded or not rather it shows how much impurity is available in your DNA as the 280 nm wavelength is absorbed by nucleic acids (DNA) and the 260 nm by secondary compounds like proteins and carbohydrates. Hence, it is advisable to check your DNA both with agarose gel and Nanodrop® spectrophotometer. Examples of Nanodrop® spectrophotometer DNA quantification are indicated in appendix 4.

Once quantified, part of the stock DNA samples were diluted with autoclaved double distilled water until the DNA concentration becomes 20 ng/μl. The diluted DNA samples were stored at -20 °C until used for AFLP analysis while the stock DNA was kept at -80 °C. A modified PCR-AFLP protocol (Vos et al. 1995) was used to assess the genetic variability of FAW populations. The AFLP procedure was completed in three basic steps: 1) DNA template preparation, 2) DNA template pre-amplification, and 3) selective amplification of the pre-amplified product.



**DNA template preparation:** The DNA samples were digested with a rare cutter, EcoRI, and a frequent cutter, MseI, restriction enzymes [New England Biolabs Ltd. (NEB), Ontario, Canada], by mixing 7 µl of the template DNA with the restriction enzymes in a sterilized PCR tube. The reaction was carried out for 2.5 h at 37°C and 15 min at 70°C by mixing the DNA template with 5.5 µl of PCR master mix containing the restriction enzymes EcoRI and MseI (both at 1.25 U/reaction), One-Phor-All buffer plus (Amersham Pharmacia Biotech Inc, NJ, U.S.A.) (1.25 µl/reaction), BSA (bovine serum albumin, NEB) (0.125 µl/reaction), and autoclaved double distilled water (3.94 µl/reaction). A detail of PCR steps is indicated in appendix 5. Then, oligotide adaptors were ligated to the restriction fragments by adding 5 µl of the adapter ligation mixture containing MseI and EcoRI adapters (0.5µl/reaction) (Integrated DNA Technologies, Iowa, U.S.A), T4 DNA ligase (NEB) (0.15 µl/reaction), T4 DNA ligase buffer (NEB) (0.5 µl/reaction) and 3.35 µl of autoclaved double distilled water. Detail of adapters is shown in Table 2.

The adapters have enzyme specific sequences that allow their ligation to the EcoRI or MseI restriction sites so that they will serve as priming sites for PCR amplification of the fragments. Moreover, the ligation of the adapters to the endonuclease restriction sites modifies the restriction sites thereby preventing further restriction to occur after ligation. The adapter ligation step was completed by incubating the restriction digest product and ligation mixture for 8 h at 25°C. The ligation product was diluted by adding 135 µl of 1X TE buffer to each sample (PCR tube).

**Pre-amplification:** Pre-amplification of the diluted ligation product was done by mixing 1.25 µl of the above adapter ligation product with 12.5 µl of pre-amplification

mix containing primer mix (1X) (10 µl/reaction) (Licor, Lincoln, Nebraska) composed of two oligonucleotide primers with EcoRI and MseI adapted ends (Table 2), 10 X PCR buffer (stoffer buffer) (1.25 µl/reaction), AmpliTaq DNA polymerase (Applied Biosystems, Carlsbad, California) (1.25 U/reaction) and 25 mM MgCl<sub>2</sub> (0.75 µl/reaction). The mixture was amplified using 20 PCR cycles of 30s at 94°C, 1 min at 56°C, and 1 min at 72°C.

The oligonucleotide primers in the preamplification mix should be complementary to the adapter/ restriction sites. The preamplification step allows the first selection of fragments by amplifying those DNA fragments that have ligated an adapter to their both ends. After completing the PCR cycles of this step, the pre-amplified product was diluted by mixing 190 µl of double distilled water with 10 µl of the preamplified product in a new PCR tube.

**Selective amplification:** To assess the genetic variability of FAW populations using AFLP, two IRD-labeled EcoRI primers (ACA and AAC) and two unlabeled MseI primers (CAA and CAG) (Licor, Lincoln, Nebraska) were selected based on preliminary primer screening experiments and used in three different combinations. Details of primer sequences and combinations are indicated in tables 3 and 4. A master mix of the selective amplification was prepared based on the amount required for a single reaction by mixing 10 x PCR buffer II (1.2 µl), AmpliTaq DNA polymerase [0.08 µl) (5 U/µl)], 25 mM MgCl<sub>2</sub> (0.72 µl), 1.5.0 µl of MseI primer, 0.3 µl of EcoRI primer and 4.7 µl of autoclaved double distilled water. Then, 8.5 µl of the master mix was dispensed into sterilized new PCR tubes and 2.0 µl of the pre-amplified product was added into the PCR tubes containing the PCR mix. This selective amplification is important to limit the number of

fragments to be amplified and label the DNA using the labeled primers (EcorI) which will help the visualization of the fragments during electrophoresis.

PCR reaction for selective amplification of the samples was done by using the "touch down" PCR program available in the Gene AMP® PCR System 2700 (Applied Biosystems, Carlsbad, California). The PCR cycles were set to 1 cycle for 30 s at 94°C, 30 s at 65°C, 1 min at 72°C; 12 cycles for 30 s at 94°C, 1min at 72°C; and 23 cycles for 30 s at 94°C, 30 s at 65°C, and 1min at 72°C. Once the PCR cycle was completed, the samples were soaked at 4°C for few minutes before the reaction was stopped. The reaction was stopped by adding 2.5 µl of blue stop solution (Licor, Lincoln, Nebraska) to each sample and the samples were denatured by holding at 95°C for 3 minutes. After finishing the denaturation step, the samples were immediately put on ice and then loaded onto the gel after cooling or stored at -20°C until they are used for electrophoresis. PCR steps after the preamplification were done in the dark and samples were covered with aluminum foil as the labeled primers are sensitive to light.

**Electrophoresis:** The electrophoresis gel was prepared by mixing 19 ml of 6.5 % polyacrylamide gel matrix (Licor, Lincoln, Nebraska), 128.4 µl of 1% ammonium-persulphate (Sigma-Aldrich, St. Louis, MO) 12.85 µl Tetramethylethylenediamin (TEMED) (Sigma-Aldrich, St. Louis, MO). The gel mix was loaded between two clean Styrofoam glasses and let to polymerize for two hours. Detail of the gel preparation is presented in appendix 5.

The electrophoresis was carried out by placing the glass plates containing the polymerized gel onto Licor Gene Read IR 4200 DNA sequencer (Licor, Lincoln, Nebraska) and adding 1 X TBE (Tris-Borate-EDTA) buffer (appendix 2) on both upper

and lower buffer tanks and then doing a 15 min prerun followed by loading 1µl of the selective amplification product of each sample in separate lanes of the 6.5% denaturing polyacrylamide gel. One µl of IRD-labeled 50-700 base pair (bp) size ladder (Licor, Lincoln, Nebraska) was loaded on the first lane and last lanes of the gel as described in Clark (2005). The marker was denatured at 95°C for 3 min and cooled on ice or stored at -20°C before used. Once loading of samples and the ladders is completed, the gel was run for 2.5 h at 45°C and 1500 volts. Images of the fragments that are migrating through the gel were collected by the camera of the Licor DNA sequencer (scanner). The TIF image of the gel was opened using the e-Squel SAGA program and saved on a computer attached to the scanner for latter scoring.

**Table 2.** AFLP primers used in adapter ligation and preamplification.

<b>Primer name</b>	<b>Primer type</b>	<b>Sequence (5'-3')</b>	<b>AFLP step</b>
<i>EcoRI</i> -F	Forward Adapter	CTCGTAGACTGCGTACC	Adapter ligation
<i>EcoRI</i> -R	Reverse Adapter	AATTGGTACGCAGTCTAC	Adapter ligation
<i>MseI</i> -F	Forward Adapter	GACGATGAGTCCTGAG	Adapter ligation
<i>MseI</i> -R	Reverse Adapter	TACTCAGGACTCAT	Adapter ligation
E(N+0)	<i>EcoRI</i> Preamp Primer	GAC TGC GTA CCA ATT C	Preamplification
M(N+1)	<i>MseI</i> Preamp Primer	GAT GAG TCC TGA GTA AC	Preamplification

**AFLP gel scoring (data collection):** A project was created in the SAGA program, the 48 wells TIF gel image was imported and the gel was calibrated by using the IRD-700 labeled 50–700 bp markers (Licor, Lincoln, Nebraska) as a reference. Once calibrated, scoring was made using the SAGA Generation 2 Software version 3.2 (Licor, Lincoln,

**Table 3.** List of primers used in AFLP selective amplification.

<b>Primer</b>	<b>Primer type</b>	<b>Sequence (5'-3')</b>
E(N+0+3)	<i>Eco</i> RI IR 700labeled Primer	GAC TGC GTA CCA ATT C <b>ACA</b>
E(N+0+3)	<i>Eco</i> RI IR 700labeled Primer	GAC TGC GTA CCA ATT C <b>AAC</b>
M(N+1+2)	<i>Mse</i> I Unlabeled Primer	GAT GAG TCC TGA GTA A <b>CAA</b>
M(N+1+2)	<i>Mse</i> I Unlabeled Primer	GAT GAG TCC TGA GTA A <b>CAG</b>

**Table 4.** Primer combinations used in AFLP selective amplification.

<b>Primer combination code</b>	<b>Primer pairs</b>
acaxcaa	E-ACA X M-CAA
aacxcaa	E-AAC X M-CAA
acaxcag	E-ACA X M-CAG

Nebraska). All gels were desmiled to guide the scoring lines (appendix 6). Visibility and sharpness of bands were used as criteria for marker selection and only the most visible and reproducible bands were selected as molecular markers for the genetic analysis. The bands were identified by scoring the presence (1) or absence (0) of the bands among the FAW populations for every selective primer combination.

A total of 221 AFLP markers per individual were scored from the three primer combinations used and the number of markers scored per primer pair varied from 69-80. Since a control sample (all 'chemicals except DNA of a sample) for each PCR step and primer combination of the different populations, if any band is detected on the control lane, bands from DNA samples on that gel similar to the band on the control lane were

discarded from scoring. The bands in the control sample could be an artifact resulted from binding of primers ('primer-dimer'). For the same primer set, the gels of all populations were scored at the same loci. Once the gels are scored, the reports were saved in PHYLIP format. The PHYLIP files were opened in NOTEPAD.

**Data analysis:** The mean coefficient of variation (% CV) based on the assessment of the errors associated with the estimation of genetic similarity was obtained after 1,000 bootstraps using the DBOOT software, version 1.1 (Coelho 2001). This helps to determine the appropriate number of loci required for acceptable precision of genetic studies in determining genetic structure and gene flow. To do the bootstrap analysis in DBOOT, the PHYLIP files of individual populations were opened in NOTEPAD, imported to Microsoft EXCEL and tab delimited, and then reopened in NOTEPAD for bootstrapping using the Jaccard coefficient available in the DBOOT program.

Once the amount of variation explained by the number of markers used is determined by plotting the coefficient of variation values against the number of markers, analysis of molecular variance (AMOVA) was performed to test the genetic structure among groups, within and among populations of FAW. Pairwise comparison was done using the ARLEQUIN program, version 3. 1, (Excoffier et al. 2005) to get the genetic dissimilarity matrix. The fixation index ( $F_{ST}$ ), which is also known as Wright's inbreeding coefficient, that measures the genetic distance between populations was also calculated as:  $F_{ST} = H_T - H_S / H_T$ , where  $H_T$  and  $H_S$  represent the average number of pairwise differences between two individuals sampled from different (between) or the same (within) population, respectively (Nei 1977).

The degree of polymorphism among groups and between populations of FAW was assessed using POPGENE version 1.32 (Yeh and Boyle 1997). Genetic variation between populations was measured using  $G_{ST}$ .  $G_{ST}$  is calculated by subtracting heterozygosity of a single population ( $H_{single}$ ) from heterozygosity of the total population ( $H_{total}$ ) and then by dividing to the heterozygosity of the total population ( $G_{ST} = (H_{total} - H_{single})/H_{total}$ ). Gene flow among FAW populations ( $Nm$ ) was also estimated using the corresponding  $G_{ST}$  as:  $Nm = 0.5(1-G_{ST})/G_{ST}$  (McDermott and McDonald 1993), where  $N$  is the number of individuals in a population and  $m$  is the proportion of those individuals resulting from immigration (Wright 1969). Generally  $Nm$  values  $> 1$  indicate the presence of significant gene flow among populations.

The POPGENE program considers heterozygosity as differences in banding patterns or polymorphisms between individuals or populations. The degree of similarity within population was assessed by creating a binary data matrix (1 or 0) of individual populations to estimate the Jaccard similarity index using the SINQUAL procedure available in the NTSYSpc-2.1 software (Rohlf, 2000). The similarity coefficient among individuals was calculated using Jaccard as:  $Jac_{ij} = a/(a+b+c)$ , where,  $a$  = number of cases where band occurs simultaneously in both individuals,  $b$  = number of cases when band occurs only in the  $i^{th}$  individual, and  $c$  = number of cases when band occurs only in the  $j^{th}$  individual. Western bean cutworm (*Loxagrotis albicosta* Smith) larvae obtained from Minnesota, United States, were used as an outlier group to test the robustness of the POPGENE analysis.

To illustrate the genetic similarity within a population, dendrograms were constructed for selected populations using SAHN-UPGMA clustering method available

in NTSYSpc described by Sneath and Sokal (1973). The correlation of geographical distance to genetic distance was estimated using the Mantel test with 1000 permutations (Mantel 1967) available in the ARLEQUIN software. Regression and correlation analysis was also performed using SAS (PROC GLM, SAS Institute 1999) and results were compared to those obtained in the Mantel test. The probability level for significant difference was set to  $P \leq 0.05$ .

## 2.4 Results

A total of 221 AFLP markers were scored from the three primer combinations used. The size of the fragments varied from 58-372 bp and the largest number of scorable AFLP bands (80 loci) were obtained from CAA(M) X AAC(E) primer combination (Table 5).

The DBOOD bootstrapping results with 1000 iterations showed that the 221 AFLP markers (per individual) analyzed in the study explained 95.6% of the variability observed in the FAW samples. In fact 165 markers were sufficient to detect 90% of the genetic variability in FAW (Fig 6). These results indicated that the molecular markers used in our study were sufficient to run further analysis to determine the genetic variability and gene flow of the FAW populations. Results from the analysis of molecular variance (AMOVA) showed that the majority (71.2%) of the total variation is within populations. The variation among FAW populations within groups accounted for 28% of the total variation and variability among groups was less than 1% (Table 6). The fixation index ( $F_{ST}$ ) which measure the genetic distance between populations was 0.288 indicating low genetic variability among populations.



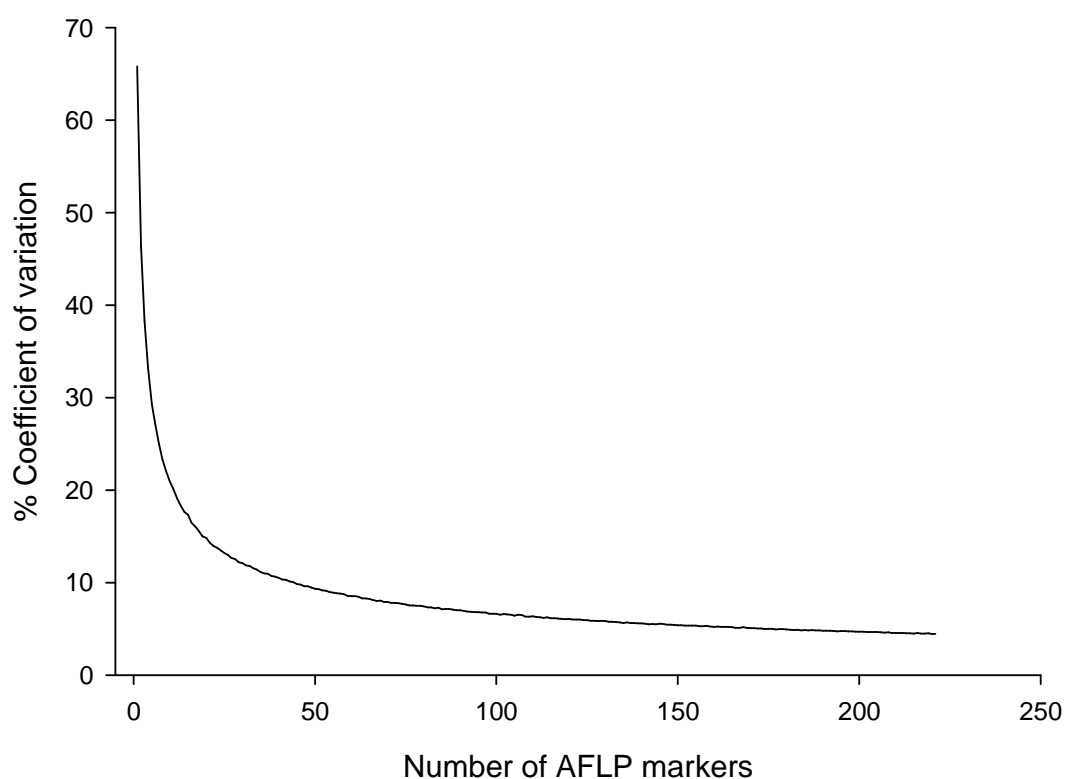
The genetic diversity estimates from POPGENE also showed that only about 27% of the variation is between FAW populations ( $G_{ST} = 0.265$ ). The number of polymorphic loci obtained from the three primer combinations for the individual populations ranged from 131 to 220 with average polymorphism of 198.2 loci for the entire population. Similarly, the average percent polymorphism was 89.7%; the lowest (59.3%) and the highest (99.5%) loci polymorphism were recorded from Argentina FAW populations collected from Ruta/SanLuis (ARG15) and Fraga/Santa Fe (ARG10) Provinces, respectively (Table 7). Moreover, the highest value for measuring genetic diversity in individual sample populations ( $H_S$ ) was recorded from TX2 and TX3 followed by ARG7 and ARG10.

**Table 5.** Selective AFLP primer combinations and associated number and size range of markers assessed in fall armyworm in the Western Hemisphere.

Primer combination	Number of AFLP markers	Fragment size (bp)
CAA(M) X ACA(E)	80	63-337
CAA(M) X AAC (E)	72	64-372
CAG(M) X ACA(E)	69	58-301
<b>Total AFLP markers</b>	<b>221</b>	

**Table 6.** Analysis of molecular variance (AMOVA) result for fall armyworm populations collected from four geographic regions in the Western Hemisphere i.e. Argentina, United States, Panama, and Puerto Rico.

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among groups	3	1326.957	0.36045 Va	0.79
Among populations within groups	27	10078.499	12.86028 Vb	28.05
Within populations	794	25899.741	32.61932 Vc	71.16
Total	824	37305.198	45.84006	



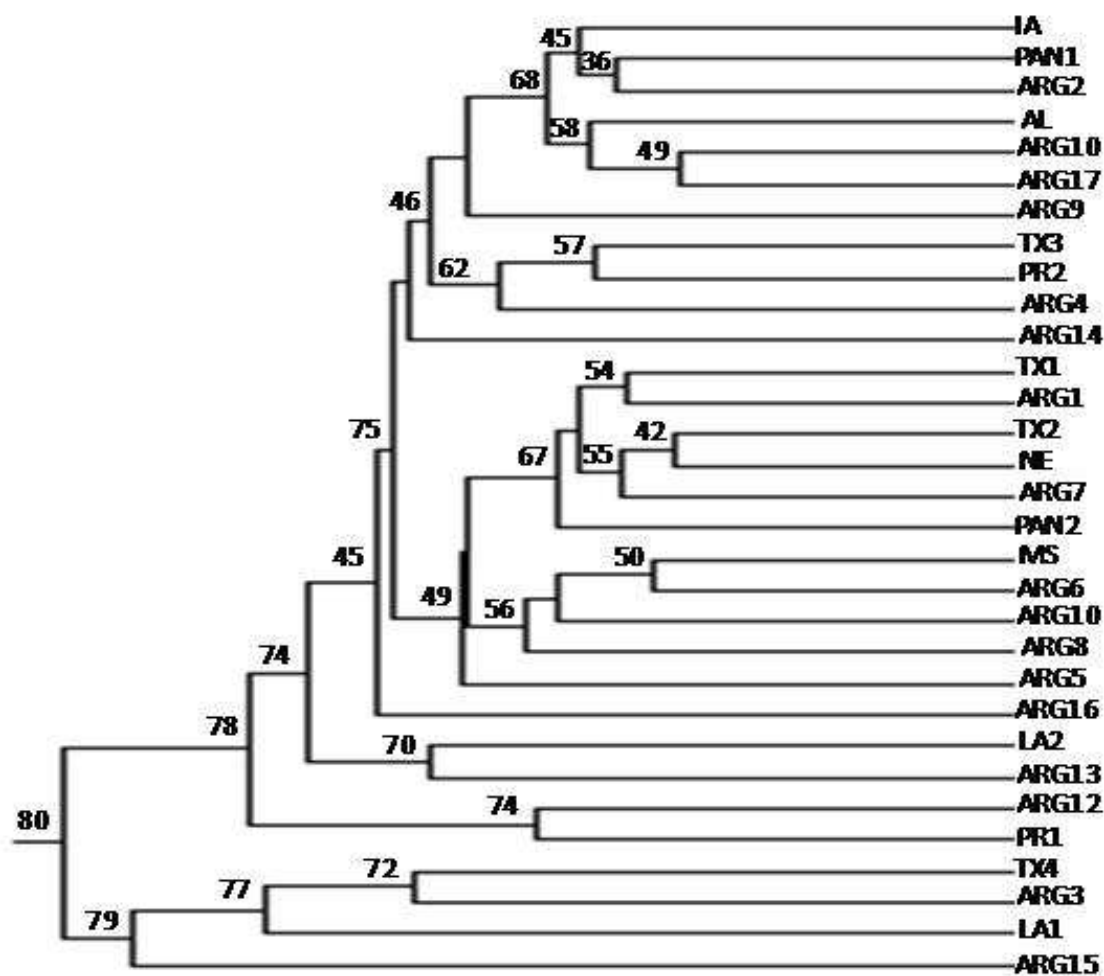
**Figure 6.** The percent coefficient of variation in relation to number of AFLP markers based on analysis of 221 loci in DBOOT with 1000 iterations for fall armyworm populations.

On the other hand, the lowest  $H_S$  value (0.2439) was observed from FAW sample collected from Iowa compared to others. The average genetic diversity index of the individual populations was 0.34901 which is higher than the  $G_{ST}$  value (0.2652) for the entire population. The  $N_m$  value for the entire populations was  $> 1$  indicating the presence of gene flow among the different FAW populations sampled (Table 7).

The dendrogram clustering in POPGENE for the entire population showed that the FAW populations clustered randomly in close proximity (Fig. 7).

**Table 7.** Genetic diversity estimates from AFLP data in 31 fall armyworm populations based on analysis of 221 loci per individual sample showing number of polymorphic loci, percent loci polymorphism, heterozygosity (genetic variation) in a single population ( $H_S$ ), heterozygosity in the entire population ( $H_T$ ), gene flow among populations ( $N_m$ ), and genetic variation between populations ( $G_{ST}$ ).

Population	# polymorphic loci	% polymorphism	$H_S$	$H_T$	$N_m$	$G_{ST}$
IA	209	94.57 %	0.2439			
AL	203	91.86 %	0.3394			
TX1	205	92.76 %	0.3984			
TX2	216	97.74 %	0.4200			
TX3	219	99.10 %	0.4248			
TX4	162	73.30 %	0.2743			
MS	191	86.43 %	0.3434			
LA1	143	64.71 %	0.2641			
LA2	199	90.05 %	0.3352			
NE	210	95.02 %	0.3750			
PAN1	212	95.93 %	0.3292			
PAN2	201	90.95 %	0.3491			
ARG1	205	92.76 %	0.3709			
ARG2	217	98.19 %	0.3325			
ARG3	179	81.00 %	0.3213			
ARG4	213	96.38 %	0.3798			
ARG5	202	91.40 %	0.3627			
ARG6	200	90.50 %	0.3802			
ARG7	213	96.38 %	0.4044			
ARG8	191	86.43 %	0.3175			
ARG9	207	93.67 %	0.3446			
ARG10	220	99.55 %	0.4081			
ARG11	182	82.35 %	0.3360			
ARG12	193	87.33 %	0.3261			
ARG13	195	88.24 %	0.3786			
ARG14	196	88.69 %	0.3460			
ARG15	131	59.28 %	0.2462			
ARG16	194	87.78 %	0.3293			
ARG17	216	97.74 %	0.3637			
PR1	203	91.86 %	0.3751			
PR2	217	98.19 %	0.3995			
Entire population average	<b>198.2</b>	<b>89.7%</b>	<b>0.34901</b>	<b>0.4750</b>	<b>1.3854</b>	<b>0.2652</b>

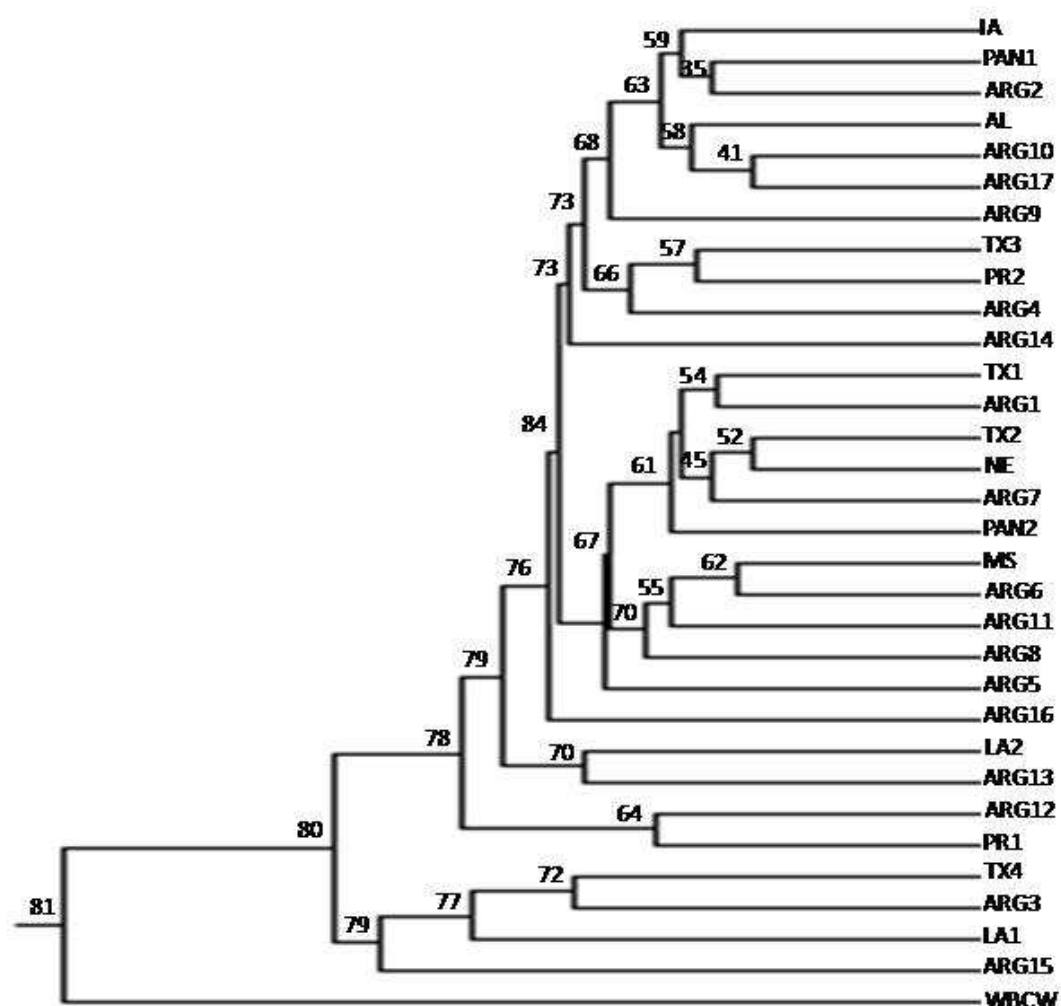


**Figure 7.** Dendrogram showing relationships and percentage similarity among 31 fall armyworm populations collected from United States (IA, LA, AL, TX, MS, and NE), Argentina (ARG1- ARG17), Panama (PAN), and Puerto Rico (PR).

Boot strap values were generally high, showing the probability of getting a similar trend in a repeated sampling. Moreover the western bean cutworm sample used as an outlier clearly separated from the FAW samples as expected, indicating the reliability of the cluster analysis (Fig. 8).

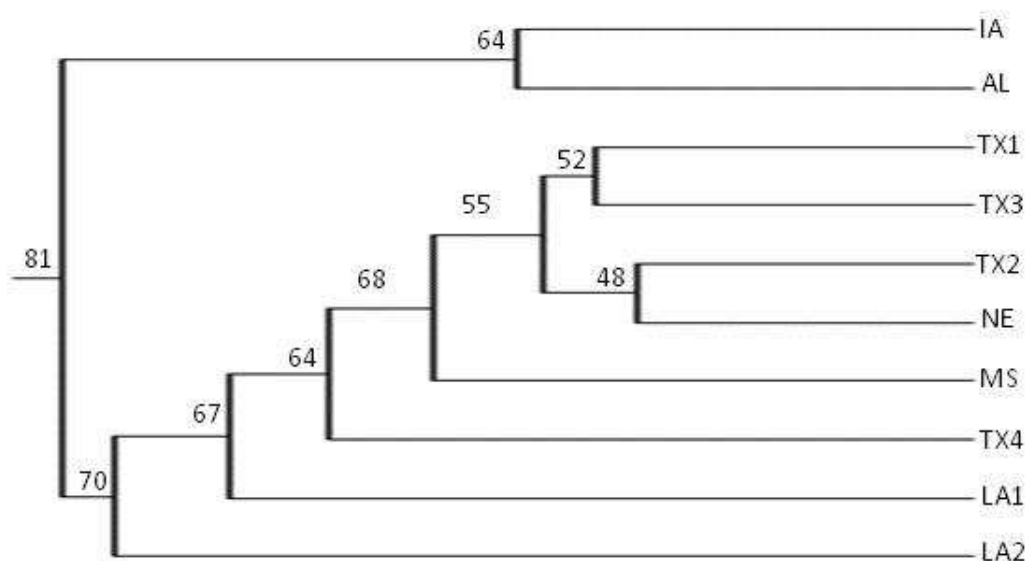
The dendrogram produced for the Argentina and United States FAW populations separately showed more variation than the close clustering observed for the all

populations. Among the U.S.A, populations, samples collected from Iowa and Alabama clustered together and showed more divergence from the rest of the populations (Fig. 9).

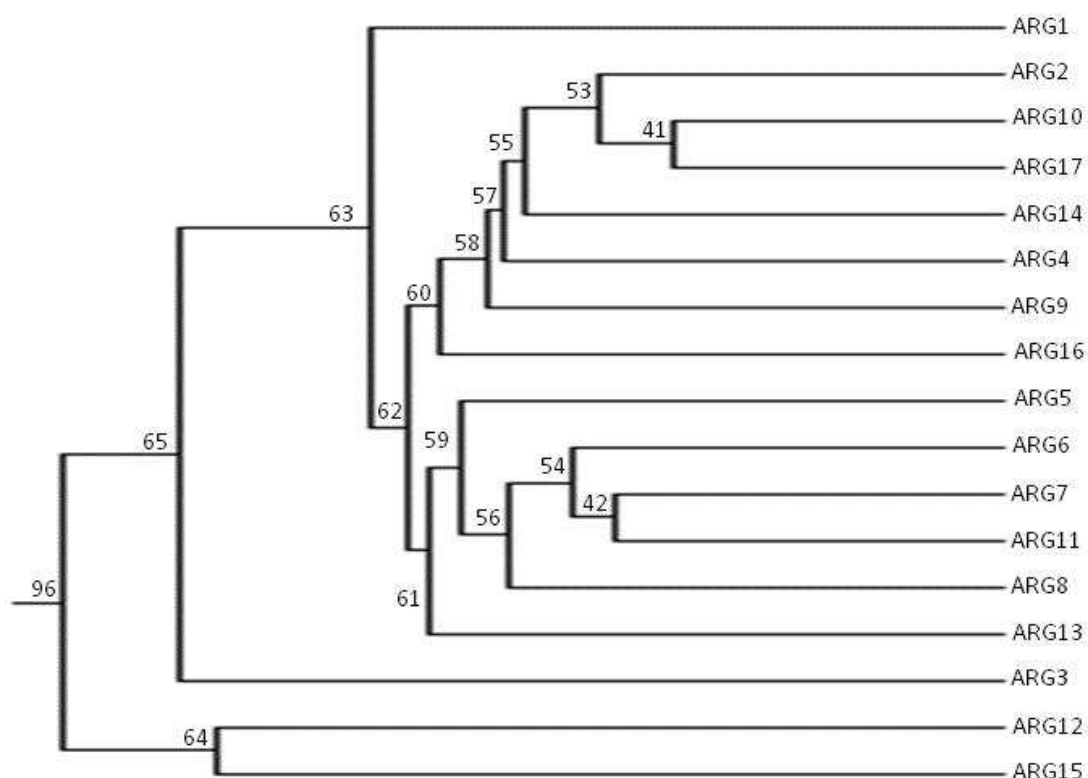


**Figure 8.** Dendrogram showing relationships and percentage similarity among 31 fall armyworm populations collected from United States (IA, LA, AL, TX, MS, and NE), Argentina (ARG1- ARG17), Panama (PAN), Puerto Rico (PR) and an out group western bean cutworm (WBCW) population collected from Minnesota.

Similarly, from the Argentina FAW populations, samples collected from Tanajera/Sgo. Del Estero (ARG12) and Ruta/SanLuis (ARG15) showed separation from other samples within the group (Fig. 10). The cluster analysis conducted with NTSYS using individuals of a given population showed less clustering and more divergence compared to the among groups and between groups analysis (appendix 11).



**Figure 9.** Dendrogram showing relationships and percentage similarity among 10 United States fall armyworm populations collected from IA, LA, AL, TX, MS, and NE based on Nei's (1978) genetic distance of UPGMA method modified from NEIGHBOR procedure of PHYLIP Version 3.5.

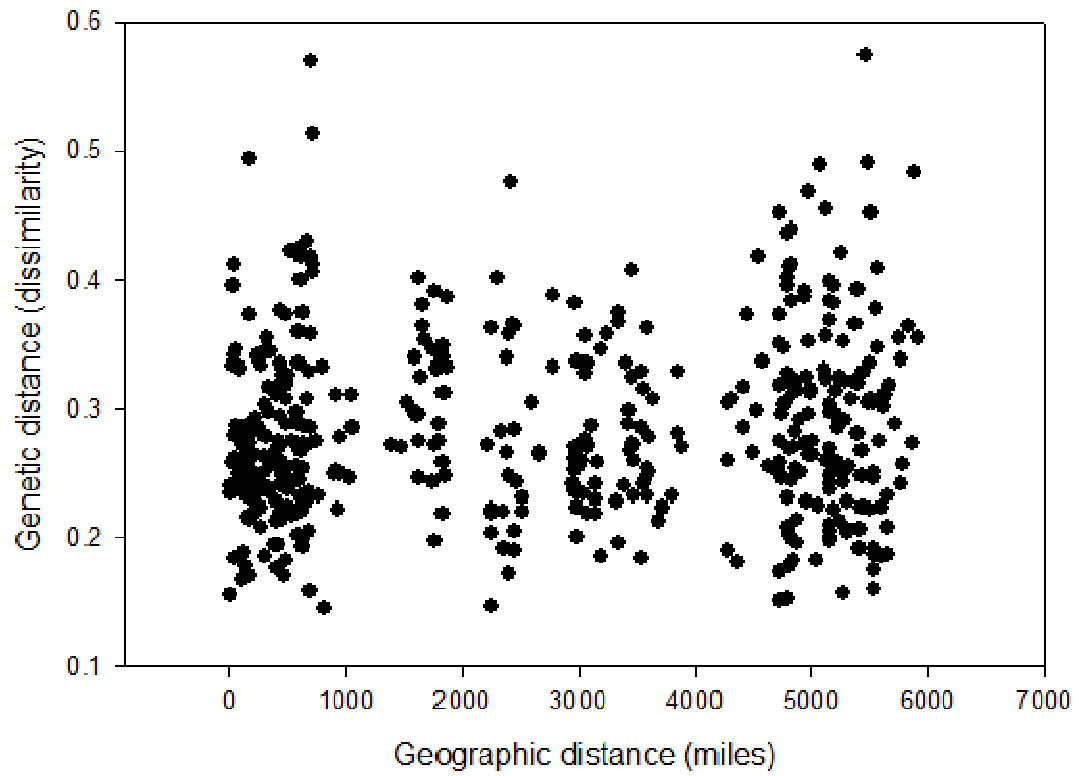


**Figure 10.** Dendrogram showing relationships and percentage similarity among 17 fall armyworm populations collected from Argentina based on Nei's (1978) genetic distance of UPGMA method modified from NEIGHBOR procedure of PHYLIP Version 3.5.

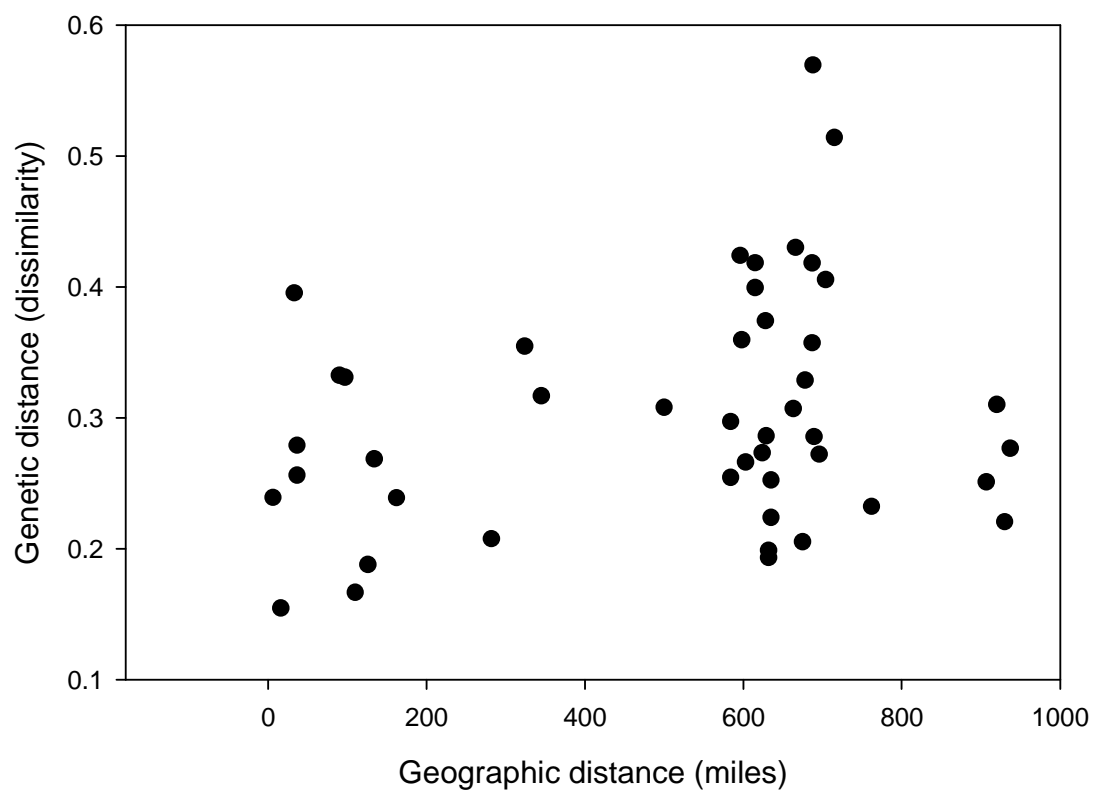
Correlation analysis using the Mantel test with 1000 iterations for the entire population revealed the absence of significant isolation by distance ( $r = 0.011014$ ,  $P = 0.06324$ ) and most of the dissimilarity matrixes lay between 0.2 and 0.3 (Fig. 11). Similarly, the regression coefficient ( $r = 0.000003$ ) shows the little dependency of genetic distance on geographic distance. The correlation and regression analysis results from SAS also showed a similar trend to that of the Mantel test. The regression of genetic distance on geographic distance was not significant ( $F_{1, 459} = 4.86$ ,  $P = 0.06328$ ,  $r = 0.10050$ ), and  $Y = 0.27484 + 0.0000346X$ , where,  $Y$  = genetic distance (dissimilarity), and  $X$  = geographic distance.

According to the Mantel test, the relationship between genetic and geographic distance for the US populations was not significant ( $r = 0.098$ ,  $P = 0.2240$ ). Similarly the SAS results indicated the lack of significant correlation between genetic dissimilarity and geographic distance ( $r = 0.23875$ ,  $P = 0.1142$ ). The regression analysis also showed that the variation based on geographic distance was not significant ( $F_{1, 43} = 2.6$ ,  $P = 0.1142$ ,  $r = 0.000076$ ), and  $Y = 0.2640 + 0.000076X$ . Moreover, the dissimilarity by geographic distance scatter plot shows random distribution of the matrixes (Fig. 12). However, FAW samples collected from Argentina showed significant isolation by distance ( $r = 0.3322$ ,  $P = 0.0300$ ). The results of the SAS output also showed a significant correlation between genetic dissimilarity and geographic distance ( $r = 0.2774$ ,  $P = 0.0011$ ) and the regression of genetic distance on geographic distance was also significant ( $F_{1, 134} = 16.66$ ,  $P = 0.0011$ , and  $Y = 0.2568 + 0.0000734X$ ). The scatter plot graph also revealed an increase in dissimilarity with increase in isolation distance (Fig.13).

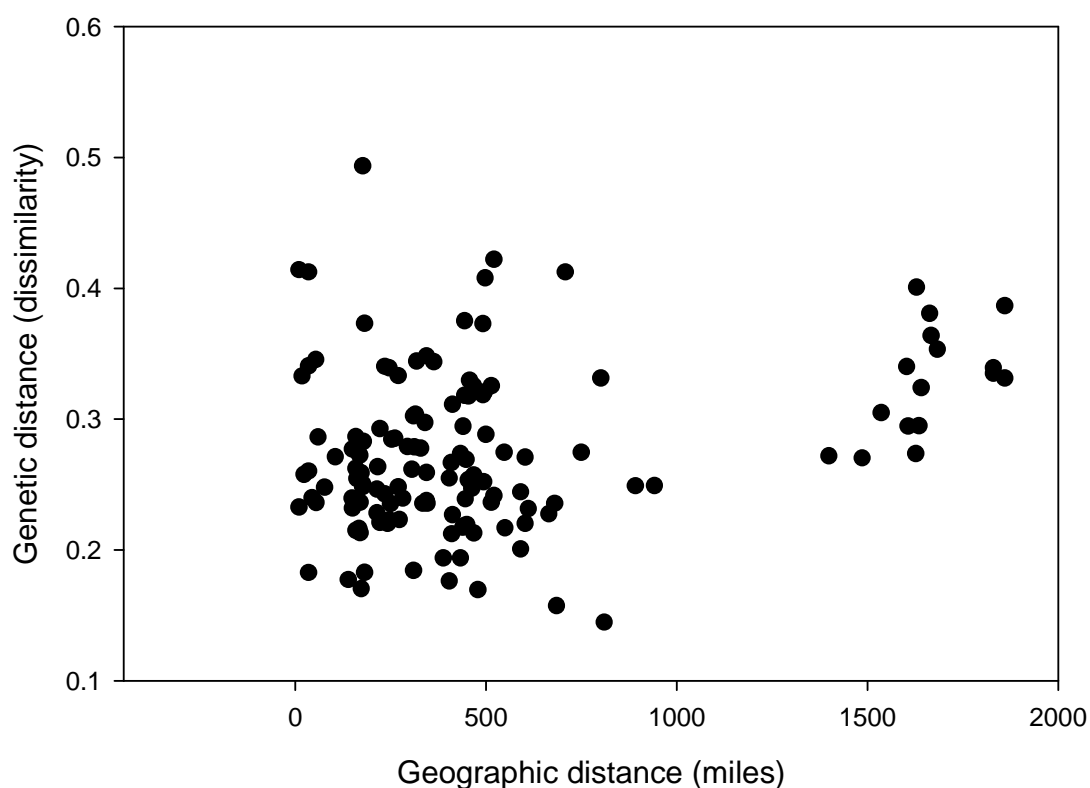




**Figure 11.** Correlation between genetic distance (dissimilarity) and geographic distance among fall armyworm populations collected from United States, Argentina, Panama, and Puerto Rico (USA) (Mantel:  $r = 0.011014$ ,  $P = 0.06324$ ).



**Figure 12.** Correlation between genetic distance (dissimilarity) and geographic distance among fall armyworm populations collected from United States (Mantel:  $r = 0.23875$ ,  $p = 0.1142$ ).



**Figure 13.** Correlation between genetic distance (dissimilarity) and geographic Distance among fall army worm populations collected from Argentina (Mantel:  $r = 0.3322$ ,  $P = 0.0300$ ).

## 2.5 Discussion

Understanding genetic diversity and gene flow of a target pest species is necessary prior to large scale efforts aimed at control of insect pests (Martinelli et al. 2007). Understanding the intraspecific level of gene flow is a fundamental step to any management practice designed to delay the evolution of resistance to any control tactics, including genetically modified crops (Roush and Daly 1990, Tabashnik 1991, Caprio and Tabashnik 1992).

With the increased introduction and adoption of Bt transgenic corn varieties in the Western Hemisphere that target lepidopteran pests including FAW, there is an increased

need to understand the genetic structure of FAW in the region (Murua et al. 2008). This kind of information is important for deployment of transgenics and designing resistance management strategies because variations in the genetic structure of a pest population in space and time and gene flow among its sub-populations are greatly responsible for the rate of resistance evolution (Fuentes-Contreras et al. 2004). Different molecular markers including AFLP have been widely used to study genetic variability and the gene flow among insect populations (Fuentes-Contreras et al. 2004, Sosa-Gomez 2004, Martinelli et al. 2006).

Although several studies have been conducted on genetic variation in FAW, most of the studies concentrated on characterizing the corn and rice strains of the pest and the geographic genetic variation were based on limited sample size and molecular markers which may not be sufficient to understand the issue of genetic structure and gene flow in FAW throughout its geographic distribution. Hence, in the present study we have investigated the genetic variability and gene flow of the corn strain in the Western Hemisphere using AFLP.

AFLP principle is based on selective amplification of restriction fragments from a restriction digest of total genomic DNA using PCR in which molecular genetic polymorphisms are identified by the presence or absence of DNA fragments. The major advantage of AFLP over other DNA-based molecular markers is its capacity to inspect an entire genome for polymorphism and reproducibility. The nuclear origin of AFLP markers is attractive because markers derived from uniparentally inherited organellar genomes (chloroplast and mitochondrial genomes) might not be sufficiently variable, or even appropriate particularly in organisms where hybridization is important (Meudt and

Clarke 2007). Hence, the methodology we used to investigate genetic variability of FAW is appropriate.

In the AFLP technique, the amplification is not completely random nor is the primer based on a known host DNA sequence. This allows for DNA fingerprinting for DNAs of any origin or complexity (Vos et al. 1995) and enables scanning the entire genome of the organism within a short period of time yielding high levels of polymorphism. Hence, AFLP provides the advantage of random amplification and repeatability to study genetic variability of insect populations including FAW.

In a population genetics study, for classical surveys of genetic diversity, population structure, and genetic relatedness, using low sample size and analyzing few markers may result in a very high sample variance and thus estimates are not reliable. However, sampling above the range does not necessarily increase the power of the analysis (Hollingsworth and Ennos 2004). This was true in our study where an increase in the number of markers has led to a decrease in the sample variance. However, increasing the number of markers after a certain point is not beneficial as the decrease in the coefficient of variation becomes insignificant. Although we have analyzed a total of 221 AFLP markers that explain 95.6% of the variation, 165 markers were sufficient to detect 95% of the variation. In general genotyping of 30 individuals and scoring of 200 or more AFLP markers is recommended to get accurate results in population genetics studies (Krauss 2000, Mariette et al. 2002). Hence, the 30 individuals and 221 markers used in the present study were sufficient to obtain reliable genetic diversity estimates of the FAW.

The majority of the genetic variation in the FAW samples that were analyzed from different geographic regions was within population; variation among populations within a region was low and variation among regions (groups) was also insignificant indicating the presence of a significant gene flow throughout the Western Hemisphere where the FAW is predominantly distributed. Supporting our findings, Clark et al. (2007) found the majority of the genetic variability in FAW samples collected from United States, Mexico, Puerto Rico, Brazil, and Argentina was within population not between populations suggesting the presence of continuous gene flow among the different geographic regions. Similar studies in other insect species also showed a higher degree of variation within populations than among populations (Coates and Hellmich 2003, Juan et al. 2004, Timmermans et al. 2005).

The cluster analysis from the POPGENE also failed to separate samples collected from United States, Panama, Argentina and Puerto indicating lack of genetic structuring of the populations and supporting AMOVA results in which the majority of the variation was between individuals within a population. Moreover, FAW samples collected from the same region (e.g. Panama or Puerto Rico) clustered more closely with samples collected from other location (Argentina or United States) indicating more variation among populations within a group (region) than among groups (Fig. 7 and 8). The AMOVA result was also substantiated in the cluster analysis for individual populations in NTSYS where more separation was observed among individuals compared to clustering of populations and groups. Although for many species genetic variability may be greater between than within populations (Roderick 1996), the within variation may be important due to local selection pressures and barriers.

The isolation by distance analysis for the entire population was also not significant, indicating the presence of gene flow among the geographic areas considered in the study and supporting the results obtained in the AMOVA and cluster analysis. Moreover, the correlation between genetic and geographic distance within the United States FAW populations was not significant further supporting the hypothesis that the source of FAW infestation in the continent is from northward annual migration of the population that overwinters in the southern part of the U.S. where it can survive mild winter climatic conditions (Nagoshi and Meagher 2004, Vickery 1929). However, the Argentina FAW populations showed a significant isolation by distance and genetic structuring indicating the presence of local barriers that limit the gene flow. Corroborating our findings, a study done by Murua and Baigoro (2004) suggested that Argentina fall armyworm populations are genetically structured.

In summary, studies conducted so far on spatial genetic variability of FAW suggested that the FAW populations in the Western Hemisphere are interbreeding and genetically heterogeneous. Hence, I have accepted the null hypothesis of no genetic difference among FAW population in the Western Hemisphere. The results of this study are important for the development of appropriate management strategies of the pest including area-wide management programs, deployment of transgenics, and resistance monitoring. In addition to geographic variation, temporal genetic variability of a pest species is important to develop appropriate pest management strategies. Hence, future genetic variation studies in FAW should consider if there is a temporal genetic variation of the pest.

**CHAPTER 3**

**SUSCEPTIBILITY OF PUERTO RICO FALL ARMYWORM (FAW)**  
**(*SPODOPTERA FRUGIPERDA* SMITH) POPULATION TO**  
**DIFFERENT INSECTICIDES**



### **3. SUSCEPTIBILITY OF PUERTO RICO FALL ARMYWORM (FAW) (*SPODOPTERA FRUGIPERDA* SMITH) POPULATION TO DIFFERENT INSECTICIDES**

#### **3.1 Abstract**

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) is the major pest of corn in Puerto Rico where several seed companies conduct both research and production activities on corn throughout the year. Insecticides are sprayed 3 times a week during the peak season to control the pest and there could be up to 25 or more sprays in one crop cycle. The presence of high pest pressure and dependency on insecticide spray to control the pest coupled with presence of multiple generations may lead to development of resistance to the insecticides. This necessitates the need to conduct a regular insecticide resistance monitoring. In this study I have investigated the susceptibility of Puerto Rico FAW population to ten different insecticides being used by Dow AgroSciences (DAS) Research Station, Santa Isabel, Puerto Rico.

The experiment was conducted using third instars of FAW in a laboratory petri dish bioassay. The results showed that rotational and mixed application of insecticides being practiced by DAS helped the FAW population in the area to be still susceptible to the insecticides tested. Radiant, Orthene, and Larvin caused > 60% FAW mortality 16 h after application followed by Tracer (40% mortality). Generally larval mortality increased with time after insecticide application and 96 h after spray, most of the insecticides gave satisfactory (> 80%) control. Moreover, the dose rate study on selected

insecticides showed that the current dose rate being used by DAS based on manufacturers' recommendation is sufficient to control the FAW.

### 3.2 Introduction

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is a polyphagous migratory pest, which is endemic to the Western Hemisphere and attacking more than 80 plant species including maize, sorghum, cotton, rice, millet, peanut, alfalfa, and other cultivated and wild plant species (Knipling 1980, Pashley 1986, Vickery 1929). Presence of multiple generations, ability to migrate and feeding on a wide host range makes FAW the most severe economic pest throughout the Western Hemisphere.

Previous studies on FAW populations in the Western Hemisphere indicated the existence of two morphologically identical host strains (corn strain and rice strain) that differ in host preference, physiology, behavior, and insecticide susceptibility (Lynch et al. 1983, Pashley 1986, Pashley et al. 1995; Prowell et al. 2004). In corn, yield losses due to FAW damage could reach up to 32% in the United States (Wiseman and Isenhour 1993) and 45-60% in Nicaragua (Hruska and Gladstone 1988, Huis 1981). Hence, insecticides are used as major components of IPM to control the pest as ability to migrate long distances and feed on a broad host range makes other control options less efficient. In the United States insecticides are applied on sweet corn against FAW often on a daily basis when the corn is at silking stage (Capinera 2001).

Although chemical insecticides can provide effective control of crop pests including FAW (Young 1979), control of FAW has been fully dependent on insecticides and as a result the pest has developed resistance to major classes of insecticides in several

locations (Yu et al. 2003). Insecticide resistance refers to a shift in the genetics of a pest population that allows individuals within a previously susceptible population to survive, because, resistant populations have inherited traits that reduce their susceptibility to individual pesticides or groups of pesticides. Development of insecticide resistance is mainly due to regular pesticide use that leads to selection of resistant individuals so that those rare individuals survive and reproduce more successfully than their susceptible peers.

Resistance development in FAW to different insecticides have been reported from Florida (Yu 1991, 1992), Argentina (Berta et al. 2000), and Honduras (Pitre 1988). In Puerto Rico, where most of the seed companies grow their corn nurseries throughout the year, FAW is the number one pest and severely reduces yield and quality without insecticide application. Therefore, insecticides are sprayed 3 times a week during the peak season and there could be up to 25 or more sprays in one crop cycle (personal communications) which places a heavy selection pressure on the pest for development of insecticide resistance. Hence, the objective of the present study was to assess susceptibility of Puerto Rico FAW population to insecticides being used by Dow AgroSciences Research Station, Santa Isabel, Puerto Rico.

### **3.3 Materials and methods**

A laboratory insecticide bioassay experiment was conducted at Dow AgroSciences (DAS) Research Station, Santa Isabel Puerto Rico in June 2010. A field population of third instar FAW was collected from corn host plants. Infested corn plants were cut from the field and brought to the laboratory and then larvae were collected by

opening the leaf sheath or dissecting stems of the corn plants, and only uniform aged larvae were retained for the experiments.

The experiment was conducted on plastic petri dishes in which a Whatman filter paper was placed before introducing the larva to avoid moisture condensation in the petri dishes during insecticide spray that may suffocate the larvae. Five larvae were placed per petri dish with a corn tissue (leaf sheath and stem) to avoid cannibalism before application of treatments. Once enough larvae were collected for the experiment, the corn tissue was removed and insecticides were directly sprayed on the larvae and then the corn tissue was placed back to the petri dishes to avoid cannibalism and death of larvae due to starvation. A total of ten insecticides were screened at the commercial (manufacturers') rate which is being used by DAS.

The Insecticides were applied using small (200 ml) plastic hand sprayers bought from local market and a separate sprayer was used for each treatment to avoid contamination of treatments. The untreated control larvae were sprayed with equal amount of water to avoid the effect of moisture difference in the petri dishes. Treatments were replicated 5 times in a completely randomized design. List of insecticides used in the experiment and rate of application is presented in table 8. Larval mortality was assessed after 16, 48, and 96 h of insecticide application and dead larvae were removed from the petri dishes during data collection. Fresh corn tissue was provided as needed for the surviving larvae until the end of the experimental period.

Four insecticides i.e. Tracer, Radiant, Orthene 97, and Larvin that showed fast killing and higher mortality of FAW in the first bioassay experiment were selected and used in a further dose rate study. Each insecticide was tested at four different rates 2x, 1x,

$\frac{3}{4}x$ , and  $\frac{1}{2}x$ , where  $x$  is the recommended dose rate being used by DAS. Similar to the previous experiment, field collected third instars of FAW were used in the dose rate study. The experimental set up and protocols were the same as the first experiment. The same sprayer per treatment was used for the four dose rates in which the lower dose rate was sprayed first. Generally larval mortality in the control treatment was very low and when cannibalism was suspected for the larval death, it was considered as missing.

**Data analysis:** Percent larval mortality was calculated using dead and alive larval counts from the treatments. All mortality values were corrected for natural mortality using Abbot's formula (Abbot 1925) as:

$$CM \% = \frac{C_A - T_A}{C_A} * 100$$

Where: CM = Corrected mortality,  $C_A$  = Control alive, and  $T_A$  = Treatment alive.

Data was analyzed using the Proc GLM procedure of SAS (SAS Institute 1999) and whenever ANOVA showed significant differences among treatments, individual treatment means were separated using Student Newman Keuls test (SNK) procedure. Data distribution was checked using the box plot method in SAS and when necessary, percent data was transformed using arcsine transformation before subjection to statistical analysis. The significance level was set to  $P = 0.05$ .

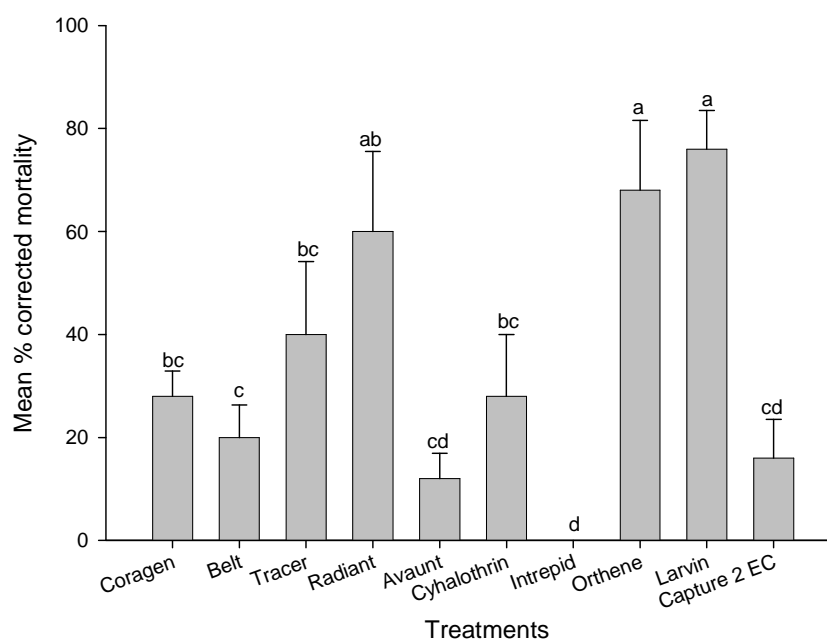
### 3.4 Results

Efficacy of insecticides tested against FAW varied with evaluation time after insecticide application ( $F_{18, 116} = 1.70$ ,  $P = 0.0485$ ). Hence, treatments were compared at specific time of mortality assessment after insecticide spray. At 16 h after application, Radiant,

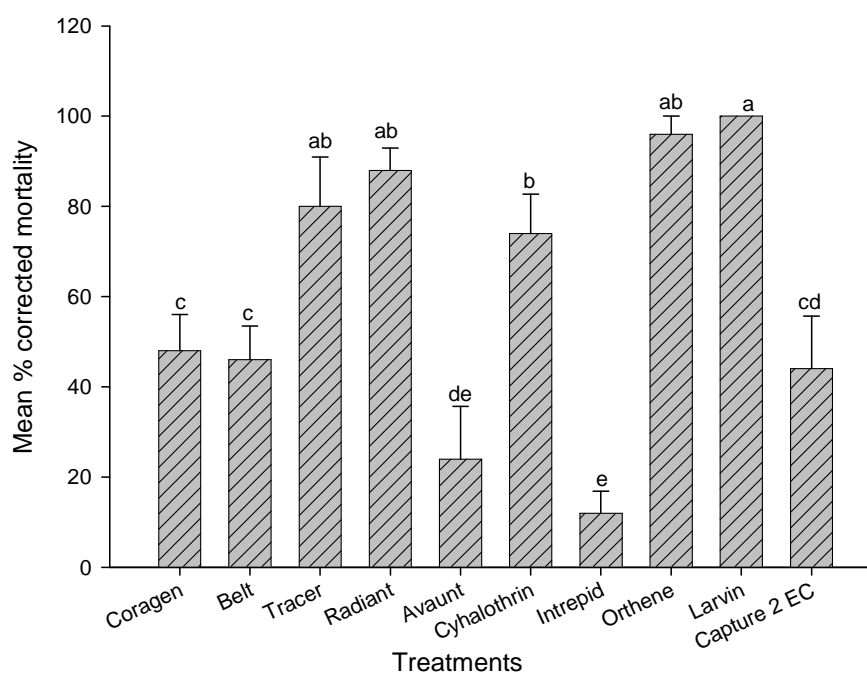
Orthene, and Larvin caused significantly higher ( $> 60\%$ ) FAW mortality compared to other treatments and Tracer was intermediate (Fig. 14). Larval mortality caused by Coragen, Belt, Cyhalothrin and Capture was low ( $< 40\%$ ) and Intrepid was not different from the untreated control. Similar trend of FAW larval mortality was observed 48 h after insecticide application (Fig. 15). Tracer showed intermediate larval mortality after 16 h of application but gave higher mortality at 48 h similar to that of Radiant, Orthene, and Larvin. Cyhalothrin also showed an increased larval mortality that was equivalent to Radiant and Orthene. However, after 96 h of application, except Intrepid and Capture, most of the insecticides caused more than 80% FAW larval mortality (Fig. 16). Tracer, Radiant, Orthene and Larvin are the most fast acting against FAW larvae (Fig. 14 and 15).

**Table 8.** List of insecticides and their active ingredient (a.i.) used in the bioassay experiment against FAW.

Insecticide name	active ingredient	Rate (oz/a)	ml/l	PPM	supplier
Coragen	Chlorantraniliprole (18.4%)	3.5	5.5	5500	DuPont
Belt SC	Flubendiamide (39.0%)	3	4.8	4800	Bayer
Tracer	Spinosad (44.2%)	2	3.2	3200	Dow
Radiant SC	Spinetoram (11.7%)	4	6.3	6300	Dow
Avaunt	Indoxacarb (30.0%)	3.5	5.5	5500	Dow
Warrior	Cyhalothrin (11.4%)	3.5	5.5	5500	Syngenta
Intrepid 2F	Methoxyfenozide (22.6%)	8	12.7	12700	Dow
Orthene 97	Acephate (97.4%)	8	12.7	12700	AMVAC
Larvin 3.2	Thiodicarb (34.0%)	30	47.6	47600	Bayer
Capture 2 EC	Bifenthrin (25.1%)	6.4	1.0	1000	

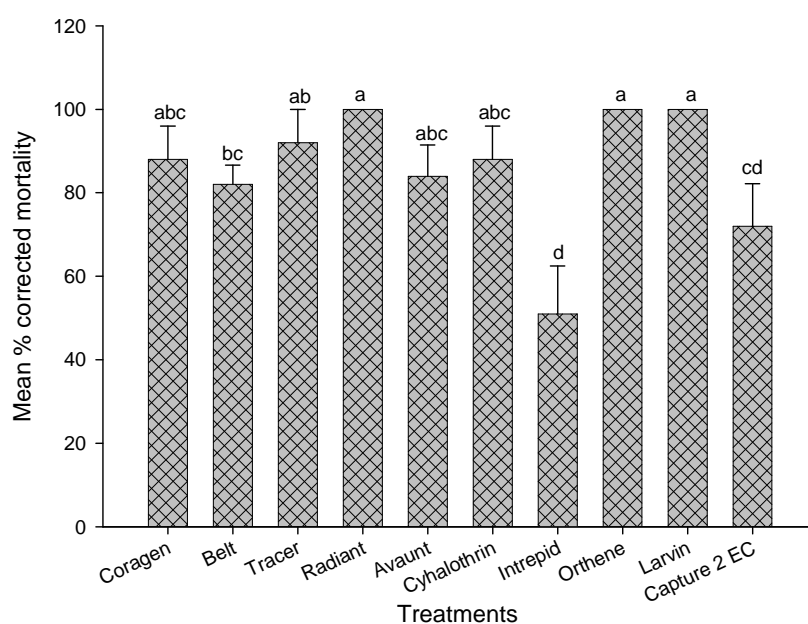


**Figure 14.** Percent FAW larval mortality 16 h after insecticide application. Means followed by the same letter are not significantly different from each other (SNK,  $P = 0.05$ ).

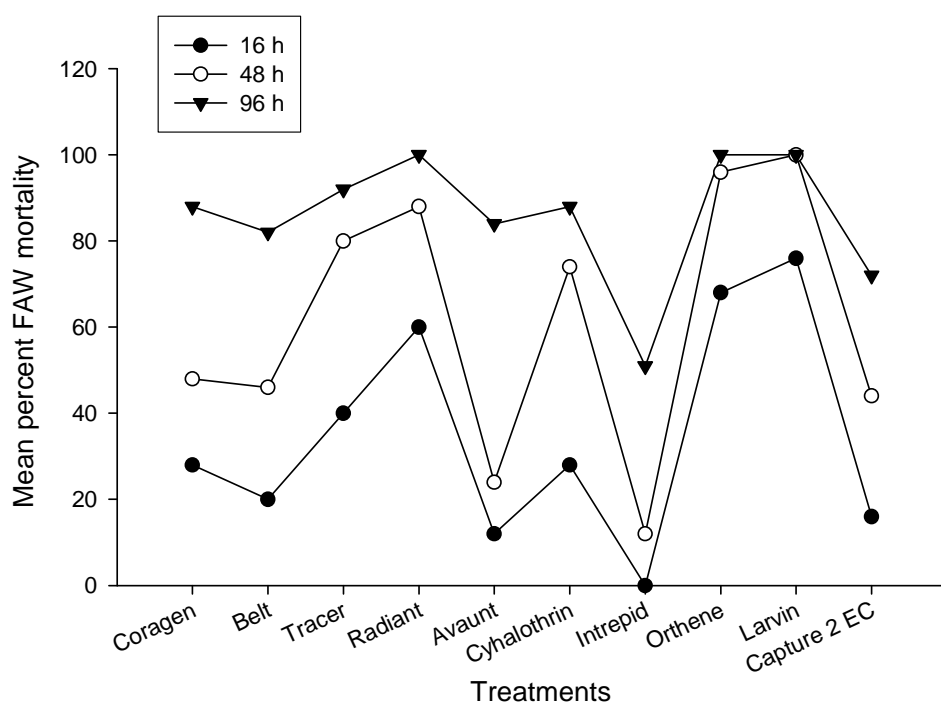


**Figure 15.**

Percent FAW larval mortality 48 h after applying different insecticides. Means followed by the same letter are not significantly different (SNK,  $P = 0.05$ ).



**Figure 16.** FAW larval mortality 96 h after applying different insecticides. Means followed by the same letter are not significantly different from each other (SNK,  $P = 0.05$ ).

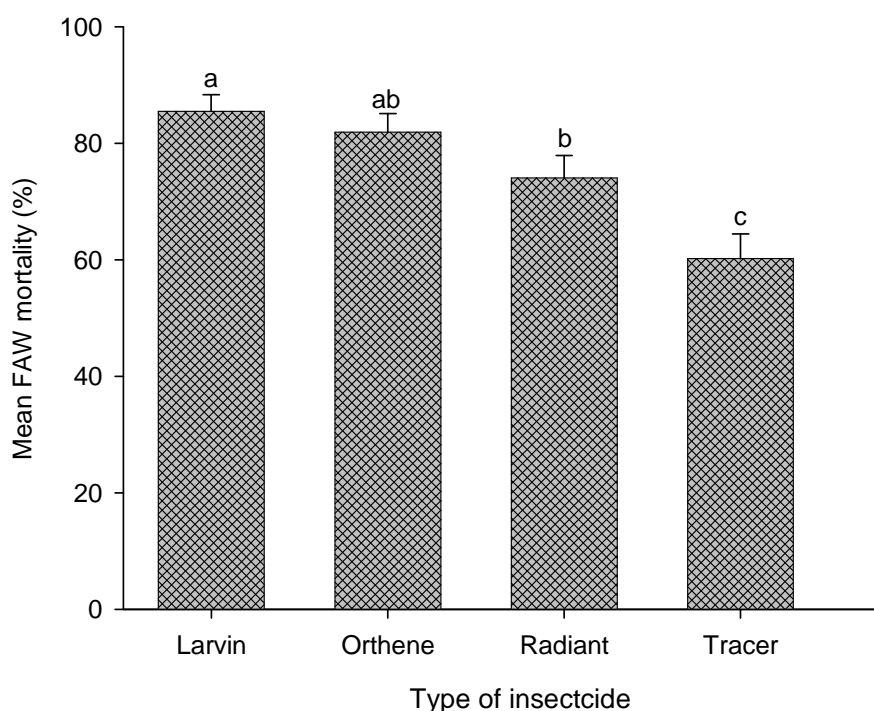


**Figure 17.** Percent corrected mortality of FAW larvae after 16, 48, and 96 h after insecticide application.



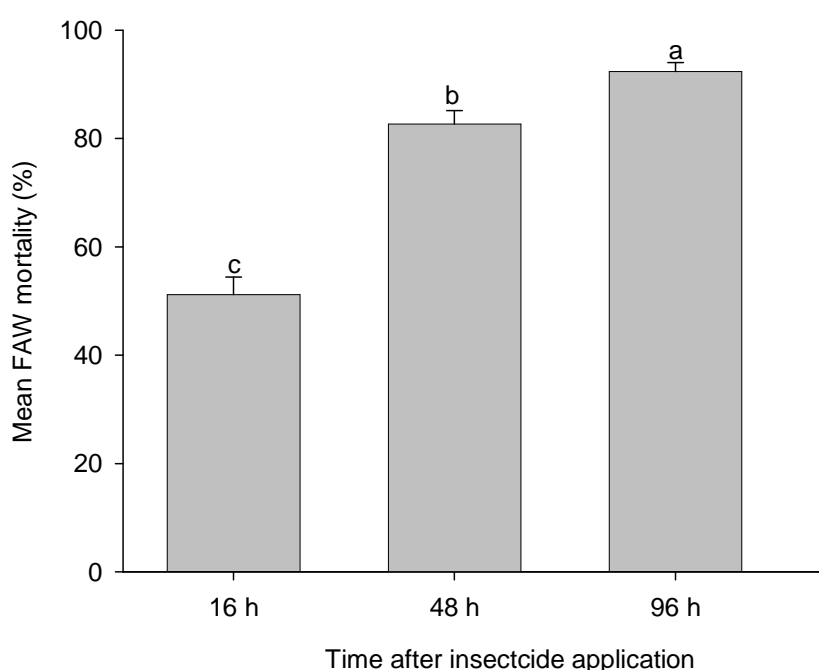
On the other hand, insecticides like Coragen, Belt, Avaunt, and Cyhalothrin caused a gradual higher mortality of FAW. Generally, for most of the insecticides tested, percent larval mortality increased with time after application (Fig. 17).

In the insecticide dose rate study, there was no significant interaction between type of insecticide, dose rate, and time of evaluation after treatment application ( $P > 0.05$ ). However, type of insecticide ( $F_{3, 161} = 15.2$ ,  $P < 0.0001$ ), time after insecticide application ( $F_{2, 161} = 57.5$ ,  $P < 0.0001$ ), and insecticide rate used ( $F_{3, 161} = 12.1$ ,  $P < 0.0001$ ), were significant. Therefore, the different factors were compared irrespective of the levels of the other factor. Among the insecticides used in the dose rate study against FAW larvae, Larvin and Orthene gave the highest mortality and Tracer was the last (Fig. 18).



**Figure 18.** Percent corrected mortality due to different insecticides. Bars followed by the same letter are not significantly different from each other (SNK,  $P = 0.05$ ).

As it is true in the first experiment, for all insecticides, percent larval mortality significantly increased with increasing time after insecticide application (Fig. 19). Overall, increasing insecticide dose rate has resulted in an increased FAW mortality (Fig. 20). However, there was no significant difference between the 2x and 1x (commercial rate) of the insecticides in terms of FAW mortality. Hence, applying Larvin, Orthene, Radiant or Tracer at the 1x rate would be sufficient to control FAW.

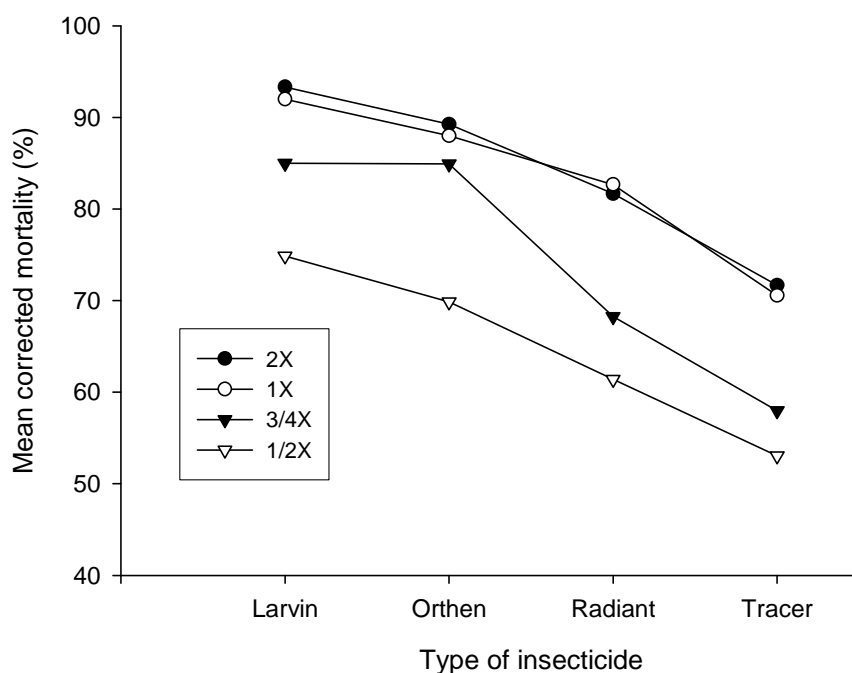


**Figure 19.** Mean FAW mortality after 16, 48 or 98 h of insecticide application. Bars followed by the same letter are not significantly different from each other (SNK,  $P = 0.05$ ).

### 3.5 Discussion

Resistance development is a major problem in many areas of pest management and by 1984 resistance to one or more insecticides had been reported in more than 447 species of insects (Georghiou and Mellon 1983, NRC 1986). Pesticide resistance management is an effort to delay or prevent the development of resistance. The resistance

management relies on pest management and pesticide use strategies to prolong the effective life of the products.



**Figure 20.** Percent FAW mortality caused by different rates of various insecticides.

Managing insecticide resistance against new and existing products involves rotating the use of products with different modes of action or applying mixture of insecticides when appropriate. Different insecticides have been used successfully to control FAW larvae on many field crops (Straub and Hogan 1974, Bass 1978, Young 1979). However, regular use and heavy selection pressure of insecticides has led to development of resistance by FAW in various regions (Yu 1991, 1992; Berta et al. 2000, Pitre 1988).

Although there is some variability in the speed of killing, most of the insecticides being used by DAS are effective in controlling FAW. The current pesticide use strategy

by DAS is based on a regular pest scouting, and rotational and mixed application of insecticides with different mode of action to control key crop pests in the area including FAW. This approach has helped to prolong the life of the products as well as to limit the number of sprays to a maximum of eight per crop cycle compared to up to 25 sprays that has been practiced before. Hence, effective pest scouting and applying insecticides in rotation or combination helped to delay development of resistance and keep the FAW population susceptible to those insecticides.

The insecticides tested in this study have shown variations in the speed of killing the FAW larvae. Some insecticides like Belt, Coragen and Intrepid have shown longer residual effects and gradual increases in insect mortality, while other like Orthene, Radiant and Larvin resulted in fast killing of the target pest. Hence, combined application of insecticide with different length of residual period as well as mode of action could provide a prolonged and effective control of target pests. Moreover, the dose rate study showed that applying Larvin, Orthene, Radiant or Tracer at the current 1x rate is sufficient to control FAW.

In insecticide resistance screening experiments, most laboratory populations lack the necessary alleles to respond due to the fact that the alleles that confer resistance are initially very rare and bottlenecks occur during laboratory colonization (Roush 1987). Hence, in the present study we used field samples of FAW that have been exposed to insecticide selection pressures instead of laboratory colony.

Further studies to determine the right time and frequency of application of those insecticides under field conditions, taking into consideration biology of the pest and Phenology of the crop, could help to achieve high level of control and prolog the

effectiveness of the products. Andrews (1980) reported that number and timing of insecticide applications was important in many areas of Central and South America which experienced economic infestations of FAW. Moreover, evaluating insecticide susceptibility of FAW populations from different regions is important as the variability of insecticide resistance/susceptibility characteristics in FAW populations may assist in determining the origin of FAW infestations and develop appropriate management strategy (Pitre 1986).

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## **APPENDICES**

### Appendix 1. FAW sample collection sites information

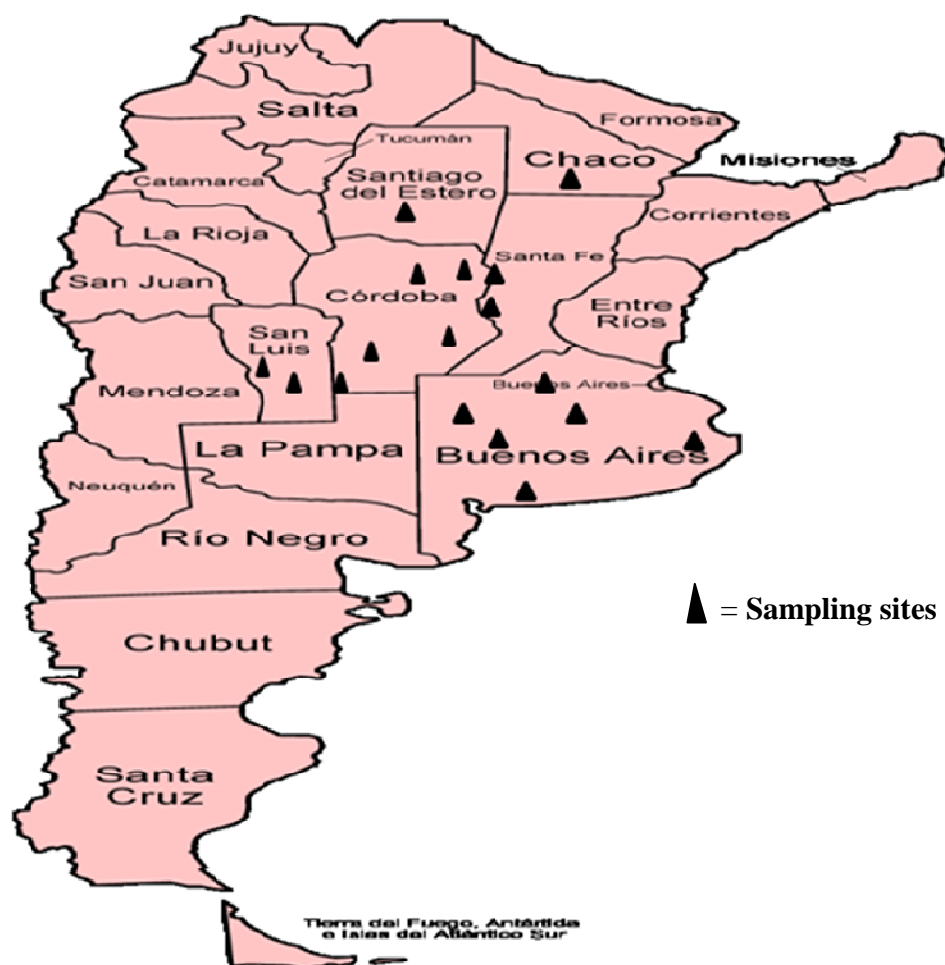


**Figure 21.** Sampling sites of US FAW populations used in the study.



▲ = Sampling sites

**Figure 22.** Sampling sites of Panama FAW populations used in the study.



**Figure 23.** Sampling sites of Argentina FAW populations used in the study.



▲ = Sampling sites

**Figure 24.** Sampling sites of Puerto Rico FAW populations used in the study.

## **Appendix 2. Research protocols used in the study**

### **2.1. DNA extraction**

The CTAB method of DNA extraction is used in the present study and the whole lab in general. FAW Larvae samples were preserved in 95 % ethyl alcohol or lyophilized and then preserved in -80°C until DNA extraction.

Procedure:

1. Place the samples into individual autoclaved eppendorf tubes and wash the samples at least two times with double distilled water.
2. Remove the gut by making a small incision with scalpel. If the larva is large, use half of the insect including head and thorax.
3. Add 250 µl CTAB buffer and homogenize with pestle manually or with a cordless pistil. Then add another 250 µl CTAB and mix well by low speed vortexing.
4. Add 10 µl proteinase K (stock conc. 20 mg/ml), vortex again at low speed to ensure proper mix of the reagents and the tissue.
5. Incubate for 1 h on 65°C heating block by gently inventing the tubes every 20 min.
6. Remove the samples from the heating block, give few minutes to cool, add 15 µl RNase A, (stock conc. 50 mg/ml) and incubate for 2 h at 37°C by gently mixing the homogenate and inverting the tubes at 20 min interval. At this stage do not vortex or you could shear the DNA.
7. Centrifuge at room temperature for 5 min at 14,000 rpm and transfer the supernatant to new autoclaved tube leaving as much debris as possible.
8. Add 500 µl chloroform: isoamyl alcohol mixture (24:1), mix it by inverting the tube several times and centrifuge for 20 min at 14,000 rpm.

9. Carefully remove the upper aqueous phase in to a new autoclaved tube and repeat the chloroform: isoamyl alcohol extraction step.
10. Transfer the upper aqueous phase again into a new autoclaved tube without disturbing the interface and add 400  $\mu$ l chilled isopropanol ( $-20^{\circ}\text{C}$ ) in to each tube. Mix gently and keep the samples at  $4^{\circ}\text{C}$  at least for 2 hrs or leave overnight the DNA to precipitate.
11. Centrifuge at 12,000 rpm for 30 min in a cooled micro centrifuge ( $4^{\circ}\text{C}$ ). At this stage a white DNA pellet will be visible at the bottom of the tube.
12. Carefully decant the supernatant, retain the pellet, add 500  $\mu$ l chilled absolute ethanol and tap the tube until the pellet comes free from the bottom of the tube and wash it by centrifuging for 5 min at  $4^{\circ}\text{C}$  and 12,000 rpm.
13. Decant the supernatant and wash pellet in cold 70% ethanol without tapping the tube. Centrifuge for 5 min at  $4^{\circ}\text{C}$  at 14,000 rpm and pour off the alcohol and retain the pellet.
14. Air dry the pellet for 30-45 min at room temperature. Once the alcohol is completely evaporated, add 50  $\mu$ l of autoclaved 1X TE buffer and resuspend overnight at  $4^{\circ}\text{C}$ . DNA can be stored short term in  $4^{\circ}\text{C}$ , but for long term storm storage, keep it either in  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$ .



## 2.2 Protocol for DNA extraction reagents preparation

### I) CTAB buffer preparation

Component	Molecular weight	Quantity needed for 200 ml
100 Mm Tris-HCL	157.59	3.152
1.4 M NaCl	58.44	16.363
0.02 M EDTA	58.44	1.489g
2% CTAB Hexa decyl trimethyl ammonium bromide)		4.00g
0.2% $\beta$ -mercapto ethanol		0.4 ml

Weigh out all the components except  $\beta$ -mercapto ethanol and dissolve in 150 ml double distilled water. Adjust the pH to 8.0 and then make up the volume to 200 ml. Autoclave the solution and add 0.4 ml (400  $\mu$ l)  $\beta$ -mercapto ethanol after cooling. Store at 4°C

**NOTE:** CTAB buffer stored at 4°C forms a precipitate, therefore before using the buffer for DNA extraction, heat the solution at a low temperature to dissolve CTAB and then use the solution.

### II) Proteinase K (stock 20 mg/ml)

Weight 0.02g Proteinase K powder in an eppendorf tube on a digital balance and dissolve it in 600  $\mu$ l of autoclaved nanopure (double distilled) water. Then add 400  $\mu$ l autoclaved glycerol. Store at -20°C.

### III) RNase A (stock 50 mg/ml)

Weight 0.05g Rnase powder. Add 600  $\mu$ l of autoclaved nanopure water. Mix thoroughly till proteinase dissolves. Then add 400  $\mu$ l autoclaved glycerol. Store at -20°C.IV).

### IV) Chloroform: Isoamyl alcohol (24:1)

Measure 240 ml chloroform into a beaker and add 10 ml iso-amyl alcohol. Mix and store in a reagent bottle at room temperature in a fume hood.

**V) Isopropanol (Iso Propyl alcohol):** Store in a bottle at  $-20^{\circ}\text{C}$ .

**VI) Absolute Ethanol:** Store in a bottle at  $-20^{\circ}\text{C}$ .

**VII) 70% Ethanol:** Mix 70 ml 99% alcohol with 30 ml with autoclaved nanopure water and store in a bottle at  $-20^{\circ}\text{C}$ .

## **2. 3. Agarose gel electrophoresis and buffers**

### **2.3.1 Gel preparation**

Depending on choice, 0.8 or 1% agarose mini-gels was used to check quality and amount of DNA in the samples. The gel can be prepared either with TBE or TAE 1X buffer and the electrophoresis should be done using the same buffer type.

- Set up the mini-gel apparatus and clean the gel tray.
- Put blocks at the ends to seal the two open ends of the gel tray.
- Clean the gel combs and insert into gel casting stand in appropriate places (negatively charged end of the apparatus).
- Weigh 1.6g agarose powder on a balance and transfer to a flask and dilute in 200 ml of 1X TAE.
- Shake the flask gently and then heat the solution in a microwave until boiling and agarose dissolves.
- Place the flask on an orbital shaker to allow gradual cooling of the gel.
- Add 12  $\mu\text{l}$  of ethidium bromide (10 mg/ml stock solution) while flask is shaking to allow thorough mixture.
- Pour the gel into the casting stand and allow to solidify (10-15 minutes).

- Add 1X TAE buffer until the gel is fully covered and remove the comb and blocks gently

**Note:** 1X TAE buffer can be used no more than 3 times. After the third run, a fresh mix of TAE buffer is prepared. Also make sure that the concentrated stock buffer did not formed precipitate, if so, discard it.

- Used ethidium bromide gel should be discarded carefully. Remaining gel can be saved and kept for another time.

### 2.3.2. Electrophoresis buffers and solutions

#### I) BUFFERS

Tris-acetate (TAE) (Agarose)	1X: 0.4 M Tris-acetate 0.001 M EDTA	50X: 242 g Tris base 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA (pH 8.0)
Tris-borate (TBE) (Polyacrylamide)	0.5X: 0.045 M Tris-Borate 0.001 M EDTA	5X: 54.0 g Tris Base 27.5 g boric acid 20 ml 0.5 M EDTA (pH 8.0)

#### II) ETHIDIUM BROMIDE SOLUTION

Preparing 10 mg/ml stock solution: **Caution** – Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye.

#### II) Gel loading dye

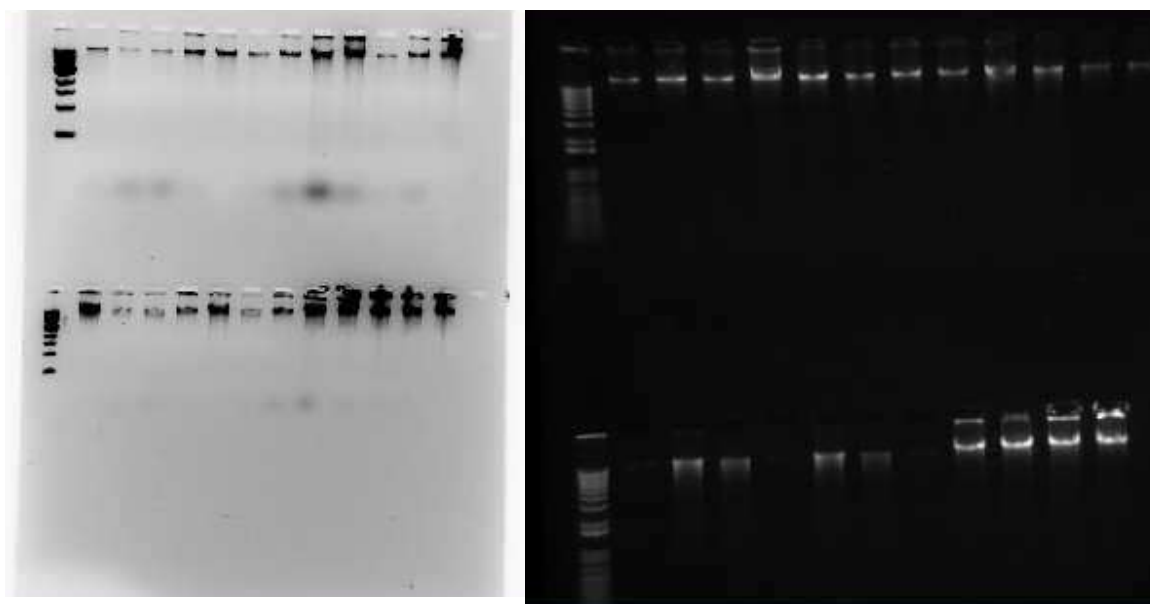
- The 6X blue loading dye consists of 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol in nanopure water.
- Store at 4 °C.
- Dilute to 1X concentration with 30% glycerol and nanopure prior to loading.

### 2.3.3 Agarose gel loading



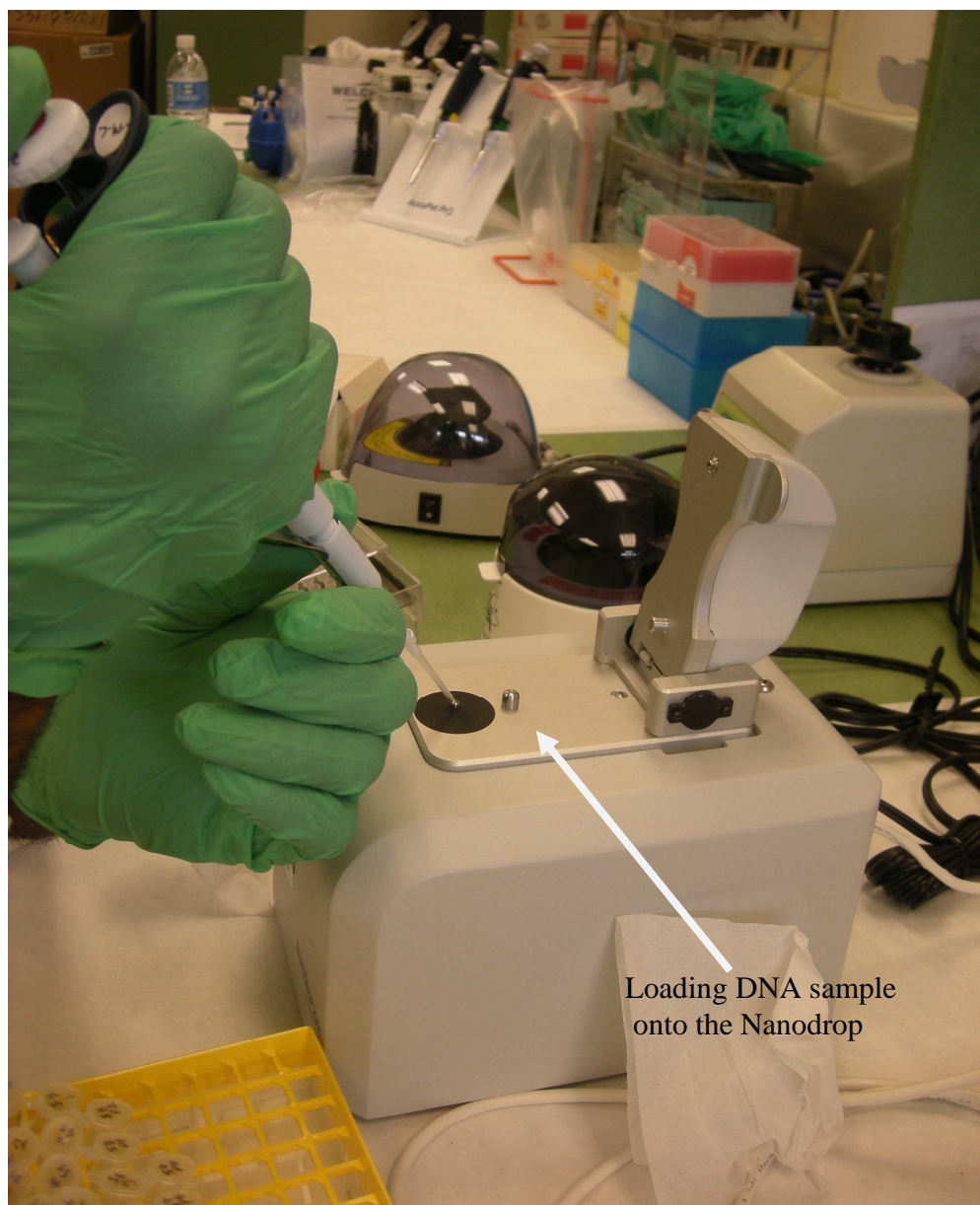
**Figure 25.** Agarose gel loading

### Appendix 3. Agarose gels



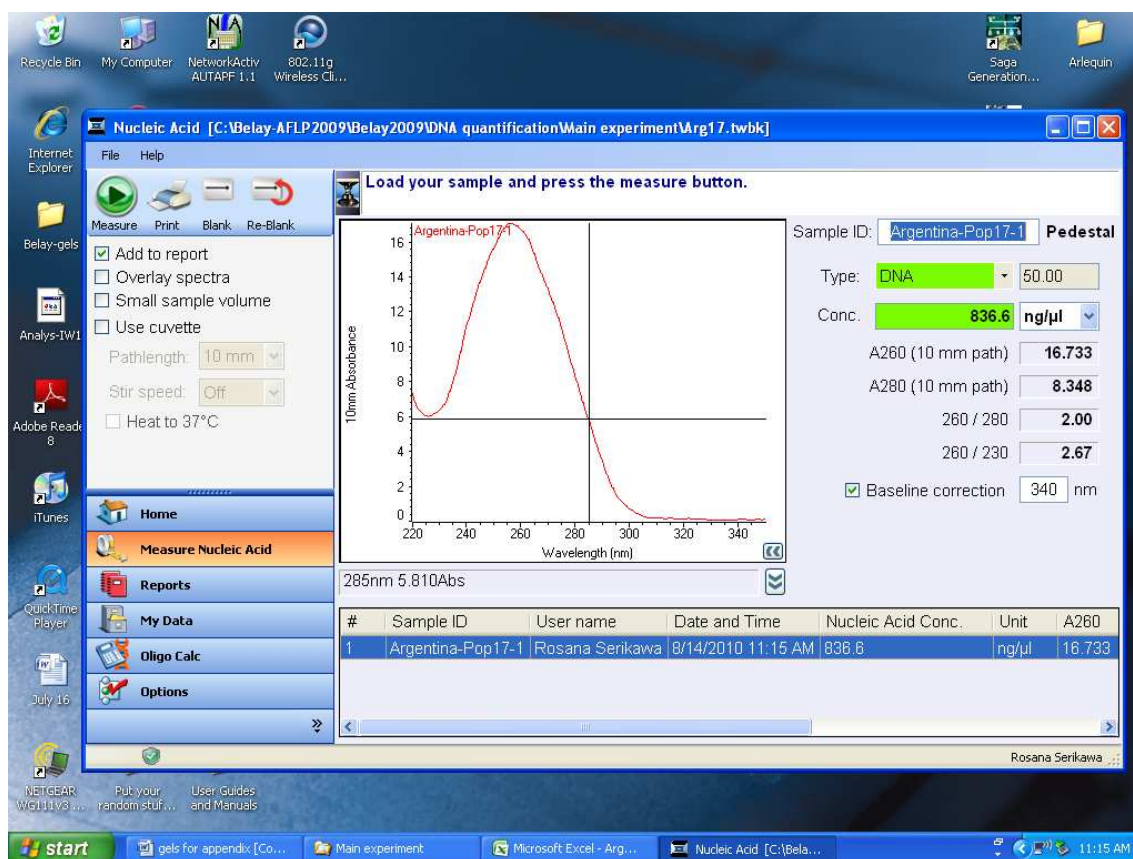
**Figure 26.** Agarose gel picture of FAW DNA.

#### Appendix 4. DNA quantification



**Figure 27.** DNA quantification with Nanodrop.

- Below is information displayed during DNA quantification using a Nanodrop Spectrophotometer.





**Table 9.** Information obtained from DNA quantification using a Nanodrop.

Sample ID	User name	Date and Time	Nuc. Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type
Arg17-1	Belay	11/28/2009	725.9	ng/μl	14.518	7.457	1.95	2.41	DNA
Arg17-2	Belay	11/28/2009	834.1	ng/μl	16.682	8.727	1.91	2.16	DNA
Arg 17-3	Belay	11/28/2009	805.6	ng/μl	16.111	8.469	1.9	1.84	DNA
Arg 17-4	Belay	11/28/2009	954.1	ng/μl	19.083	9.791	1.95	2.29	DNA
Arg 17-5	Belay	11/28/2009	556	ng/μl	11.119	5.717	1.94	2.29	DNA
Arg 17-6	Belay	11/28/2009	724.7	ng/μl	14.494	7.441	1.95	2.32	DNA
Arg 17-7	Belay	11/28/2009	586.5	ng/μl	11.73	6.131	1.91	1.69	DNA
Arg 17-8	Belay	11/28/2009	288.4	ng/μl	5.767	3.163	1.82	1.38	DNA
Arg 17-9	Belay	11/28/2009	81.8	ng/μl	1.636	0.847	1.93	2.17	DNA
Arg 17-10	Belay	11/28/2009	161	ng/μl	3.22	1.672	1.93	2.19	DNA
Arg 17-11	Belay	11/28/2009	563.8	ng/μl	11.277	5.793	1.95	2.24	DNA
Arg 17-12	Belay	11/28/2009	1042	ng/μl	20.84	10.63	1.96	2.2	DNA
Arg 17-13	Belay	11/28/2009	619	ng/μl	12.381	6.385	1.94	2.3	DNA
Arg 17-14	Belay	11/28/2009	479.7	ng/μl	9.594	4.94	1.94	2.28	DNA
Arg17 -15	Belay	11/28/2009	329.2	ng/μl	6.583	3.423	1.92	2.09	DNA
Arg 17-16	Belay	11/28/2009	442.5	ng/μl	8.849	4.653	1.9	1.39	DNA
Arg 17-17	Belay	11/28/2009	214.6	ng/μl	4.292	2.29	1.87	1.45	DNA
Arg 17-18	Belay	11/28/2009	1199.8	ng/μl	23.996	12.555	1.91	1.88	DNA
Arg 17-19	Belay	11/28/2009	615	ng/μl	12.3	6.327	1.94	2.38	DNA
Arg 17-20	Belay	11/28/2009	799	ng/μl	15.981	8.216	1.95	2.46	DNA
Arg 17-21	Belay	11/28/2009	574.3	ng/μl	11.486	5.683	2.02	2.36	DNA
Arg 17-22	Belay	11/28/2009	367.9	ng/μl	7.359	3.606	2.04	2.25	DNA
Arg 17-23	Belay	11/28/2009	669.6	ng/μl	13.392	6.633	2.02	2.34	DNA
Arg 17-24	Belay	11/28/2009	533.5	ng/μl	10.67	5.224	2.04	2.23	DNA
Arg 17-25	Belay	11/28/2009	602.2	ng/μl	12.044	5.8	2.08	2.34	DNA
Arg 17-26	Belay	11/28/2009	1154.6	ng/μl	23.093	11.486	2.01	2.33	DNA
Arg 17-27	Belay	11/28/2009	753.5	ng/μl	15.071	7.453	2.02	2.42	DNA
Arg 17-28	Belay	11/28/2009	601.4	ng/μl	12.029	6.047	1.99	2.23	DNA



## Appendix 5. AFLP steps

### Step1. Restriction digest

Component	Stock concentration	Concentration needed	Volume/ 1 reaction (µl)
One-Phore All buffer	10X	1X	1.25
MseI enzyme	4U/ µl	1.25U	0.125
EcoRI enzyme	15U/ µl	1.25U	0.0625
BSA	10mg/ml	1.25 µg	0.125
Autoclaved double distilled water	-	-	3.94

- Prepare a master mix enough for your samples and dispense 5.5 µl of the mix into each PCR tube
- Add 7 µl template DNA (20ng/ µl )
- PCR cycle: 37°C for 2.5 hr, 70°C for 15 min, and you can soak at 4°C.

### Step 2. Adapter Ligation

Component	Stock concentration	Volume/ 1 reaction (µl)
MseI enzyme	10X	0.5
EcoRI enzyme	10X	0.5
T <sub>4</sub> DNA ligase buffer	10X	0.5
T <sub>4</sub> DNA ligase	400U/ µl	0.15
Autoclaved double distilled water	-	3.35

- **Dispense 5.0 µl** of the ligation mix into each PCR tube containing restriction digest product.
- PCR cycle: Run for 8 hrs at 25°C. You can soak at 4°C.

**Step 3. Diluting the ligation product:** Dilute to 1:10 by adding 135  $\mu$ l 1X autoclaved TE buffer in to each PCR tube containing the ligation product.

**Step 4. Preamplification (modified from Licor prptocol)**

Component	Stock concentration	Concentration needed	Volume/ 1 reaction ( $\mu$ l)
Preamp primer mix	10X		10
10X PCR buffer	10X	10X	1.25
MgCl <sub>2</sub>	25 mM	25 mM	0.75
AmpliTaq DNA polymerase	10U/ $\mu$ l	1.25U	0.25

- Dispense 12.25  $\mu$ l of the master mix into new PCR tubes and add 1.25  $\mu$ l of the diluted template DNA (Ligation product).
- PCR cycle: 20 cycles of 94°C for 30 sec, 56°C for 1 min, 72°C for 1 min.  
You can soak at 4°C.

**Step 5. Checking preamplified DNA on agarose gel**

- Prepare 0.8 or 1% agarose gel.
- Mix 1  $\mu$ l of the preamplified product with 1  $\mu$ l of gel loading dye and load the mix into a separate lane.
- Run the gel for 15 min at 60 volt.

**Step 6. Dilution of the preamplified DNA template:** Mix 190  $\mu$ l of autoclaved double distilled water and 10  $\mu$ l of the preamplified product into new PCR tubes (1:20 dilution).

**Step 7. Selective amplification**

- Prepare a master mix as indicated in the following table.

- Dispense 8.5  $\mu\text{l}$  of the mix into new PCR tubes and add 2  $\mu\text{l}$  of the diluted preamplified template DNA from step 6.

Component	Stock concentration	Concentration needed	Volume/ 1 reaction ( $\mu\text{l}$ )
10X PCR buffer	10X	10X	1.2
MgCl <sub>2</sub>	25 mM	25 mM	0.72
AmpliTaq DNA polymerase	10U/ $\mu\text{l}$	1.25U	0.08
MseI primer	-	-	1.5
EcorI primer	-	-	0.3
Autoclaved double distilled water	-	-	3.94

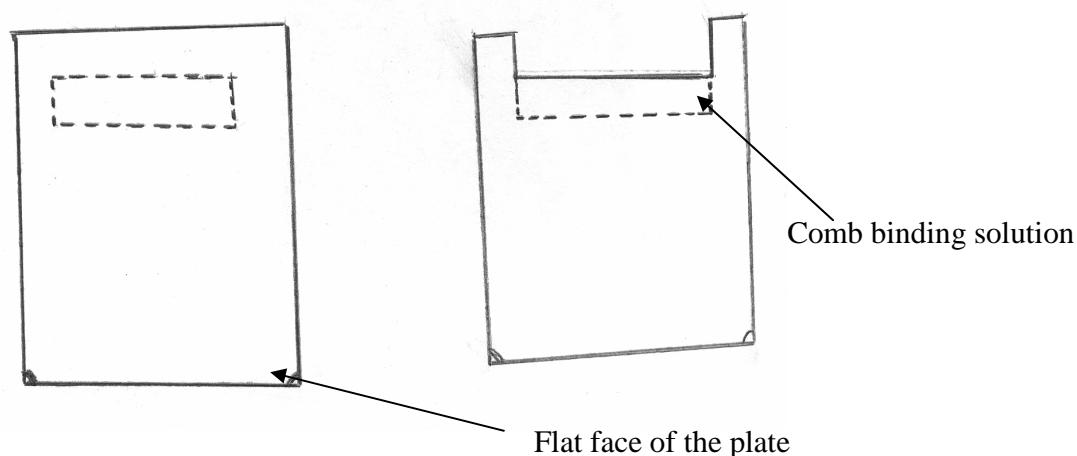
- PCR cycles:
  - 1 cycle
    - 94°C for 30 sec
    - 65°C for 30 sec
    - 72°C for 1 min
  - 12 cycles
    - 94°C for 30 sec
    - 56°C for 30 sec
    - 72°C for 1 min
  - 23 cycles
    - 94°C for 30 sec
    - 56°C for 30 sec
    - 72°C for 1 min

**Step 8. Stop the reaction.**

- Stop the PCR reaction by adding 2.5  $\mu$ l blue stop solution to each PCR tube.
- Denature the AFLP product by running 1 PCR cycle at 94°C for 2 min.

**Step 9. Polyacrylamide preparation and electrophoresis**

- Align the gel casting plates properly with the flat side up (see diagram of plates).
- Apply a solution of 100  $\mu$ l of binding silane + 100  $\mu$ l of 10% acetic acid in broken line region as shown below.



**Figure 28.** Diagram of AFLP stair foam glass plates used in AFLP electrophoresis.

- Align the spacers (0.2mm) and join the plates using the casting stand to allow for proper fit into the LI-COR 4200 sequencer. Do not over-tighten the gel casting as to not crack the plate. They only need to be tight enough to stick together.
- Prepare the polyacrylamide by placing 19 mls of KB<sup>Plus</sup> 6.5% ready made gel matix (LI-COR) into a small beaker. Add 128  $\mu$ l of ammonium persulfate

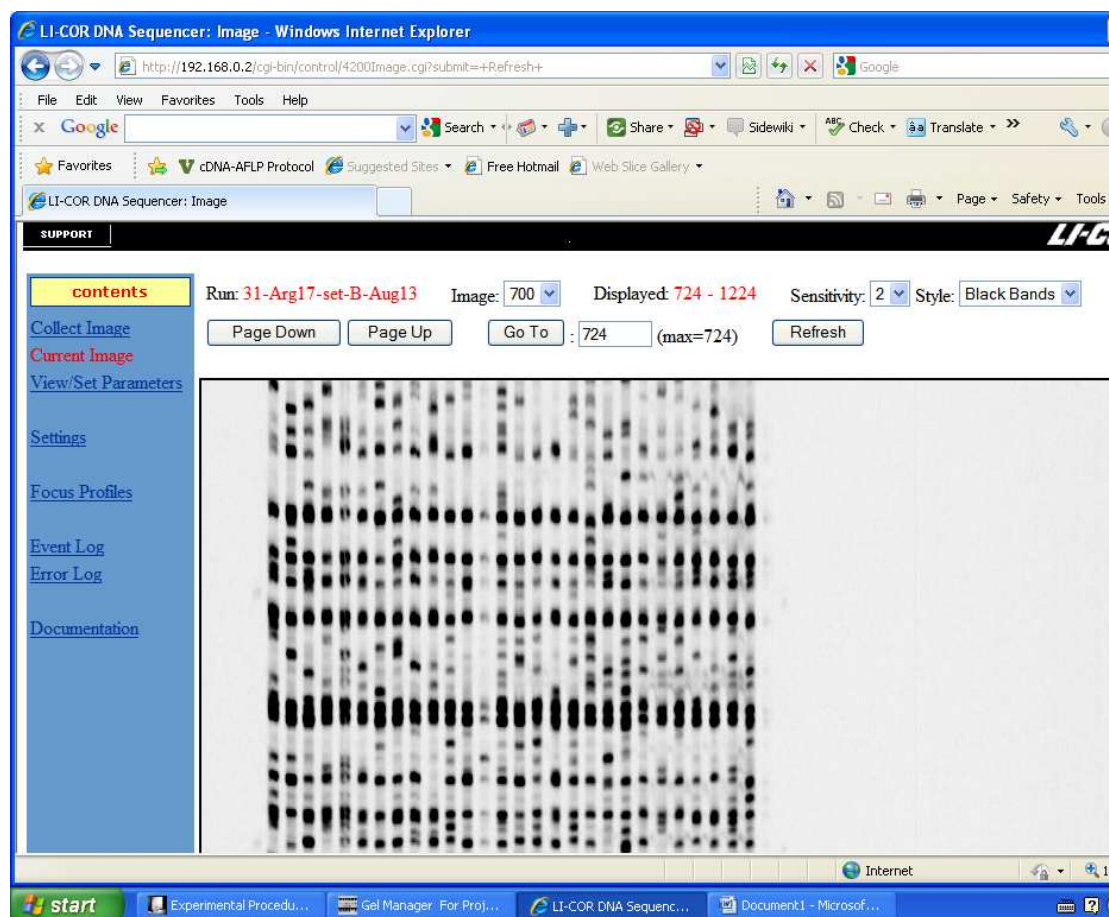
solution into the beaker containing the ready made gel (place ~ 0.0150g of APS/150 µl of nonopure water and mix by vortexing thoroughly). Place 12.8 µl of TEMED into the beaker and continue to stir.

- Pour the gel matrix into the gel casting (placed on the stand horizontally) using a pipette.
- Insert the comb being very careful not to bend the comb teeth. Place a little gel that is left over around the comb securing good well morphology.
- Place the casting plate over the glass to insure the plates are tight around the comb.
- The gel will be ready for use after 2.0 hrs.
- Using a squirt bottle, place large amount of water between the comb and plate. Gently scrape away any excess gel behind the comb to allow the comb to slide out. Gently slide the comb out of the plates allowing the water to fill in where the comb was so there are no bubbles in the wells.
- Gently clean the plates with nanopure water disposing of any towels into an acrylamide waste container. Place the casting sandwich into the casting stand and rinse with iso-propanol and allow drying. This will displace any remaining water left on the sandwich. The casting stand is then ready to be placed into the sequencer.
- After the gel has run for 2.5 h (up to 3 runs can be made with each gel), click on done collecting and then the casting stand can then be removed from the sequencer and separated using a plastic wedge inserted where there is a flat face on the glass. After the plates have separated, the gel can then be disposed

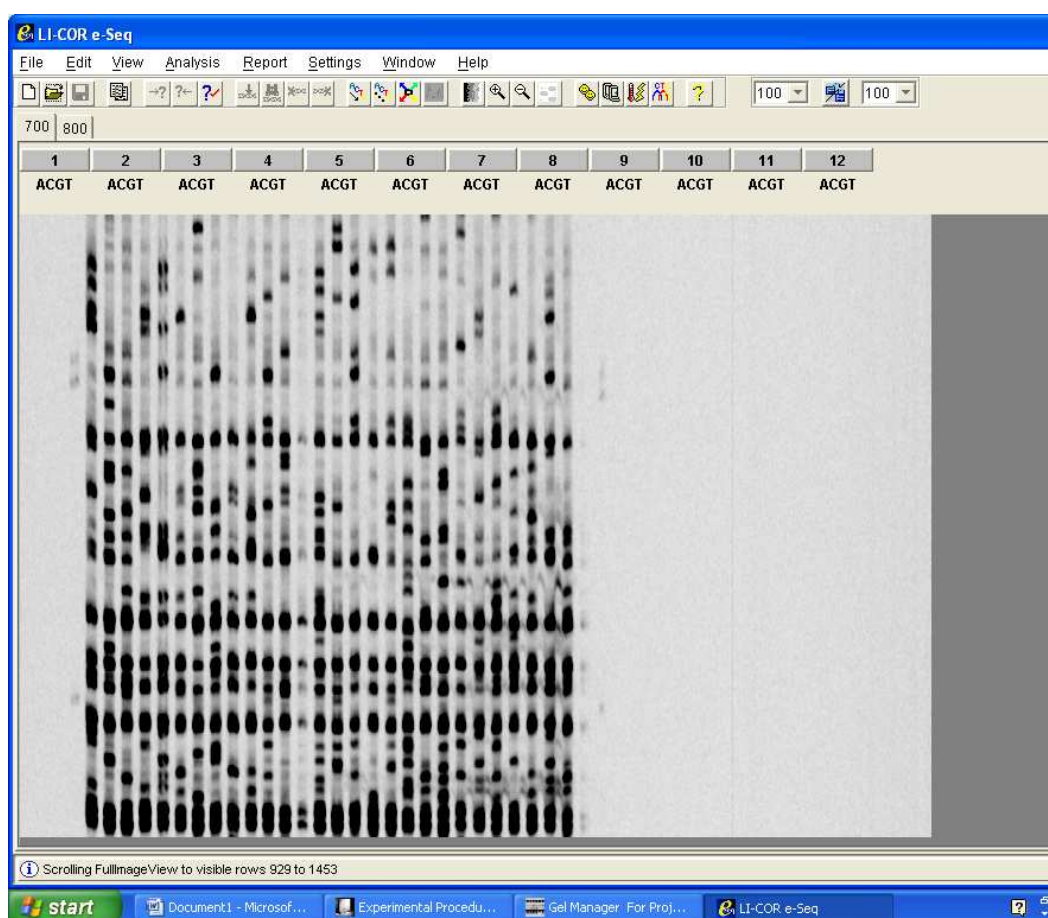
of by placing paper towels flat on the glass. Press with the hand on the towel a couple times ensuring the towel will pick up the gel. To pick up the gel, gently pull up the towel on one side slowly towards the other. The gel should stay attach to the towel.

- After the gel is removed from the plates, they can then be washed with a gentle detergent (10% viomex) and water. The plates must be completely clean.
- After they are clean they can be placed in a stand and rinsed with isopropanol to dissipate any water (water causes spots and spots causes bubbles in the gel). Allow plates to air dry.

## Appendix 6. AFLP gels

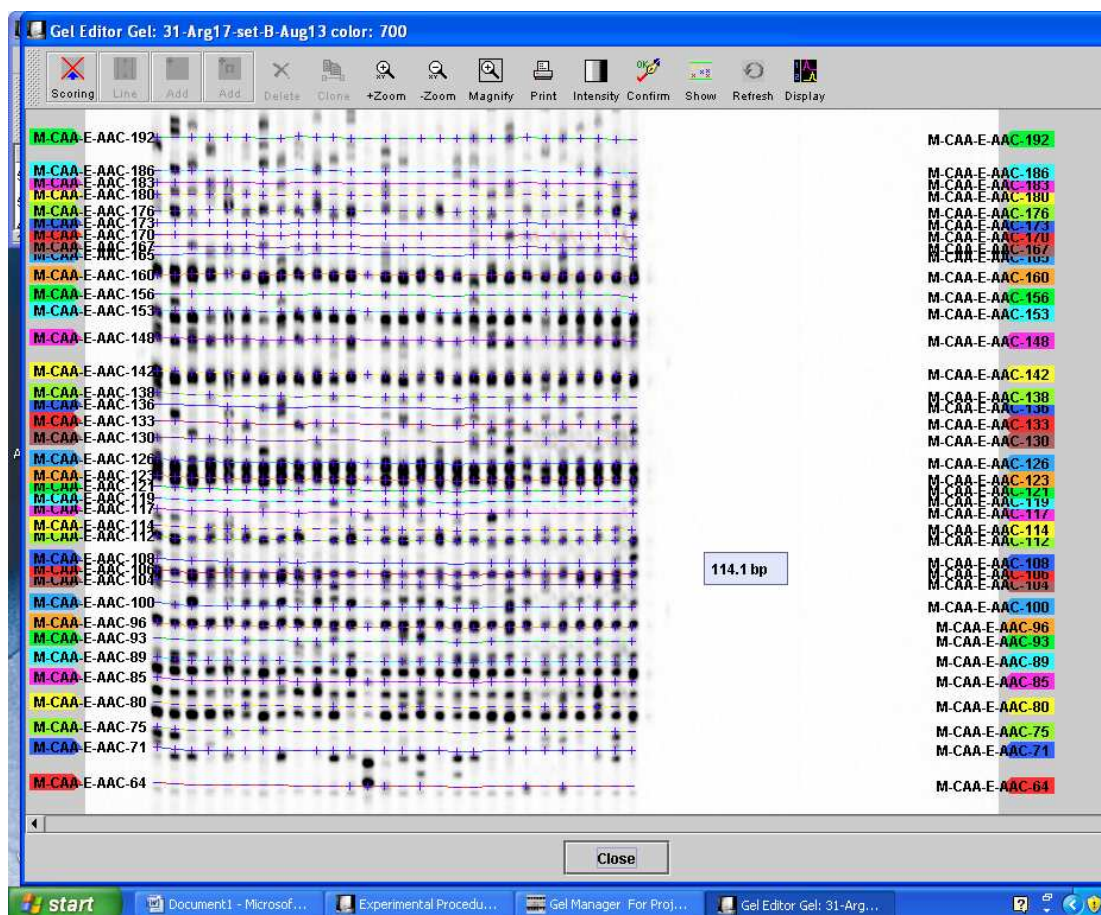


**Figure 29.** AFLP gel picture running in a licor DNA sequencer.



**Figure 30.** AFLP gel opened from the scanner into e-Sequel program.

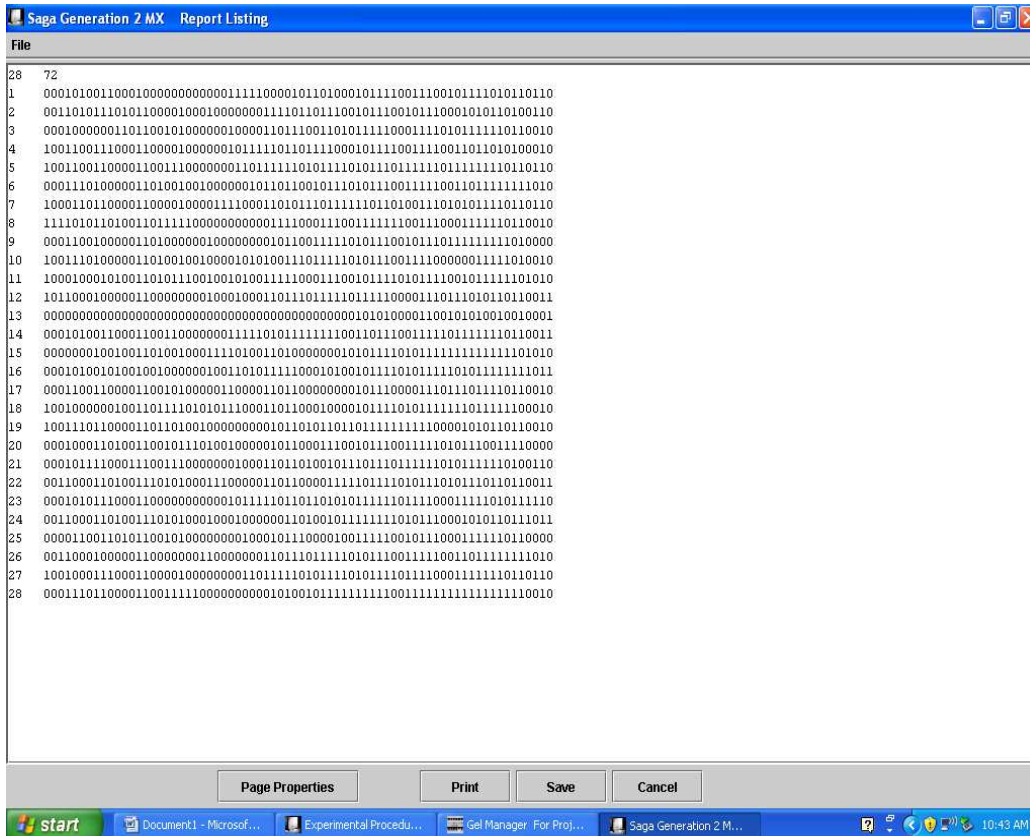




**Figure 31.** Calibrated, decimiled, and scored AFLP gel picture in SAGA.

## Appendix 7. SAGA data format

- Below is an example of AFLP data generated by SAGA.



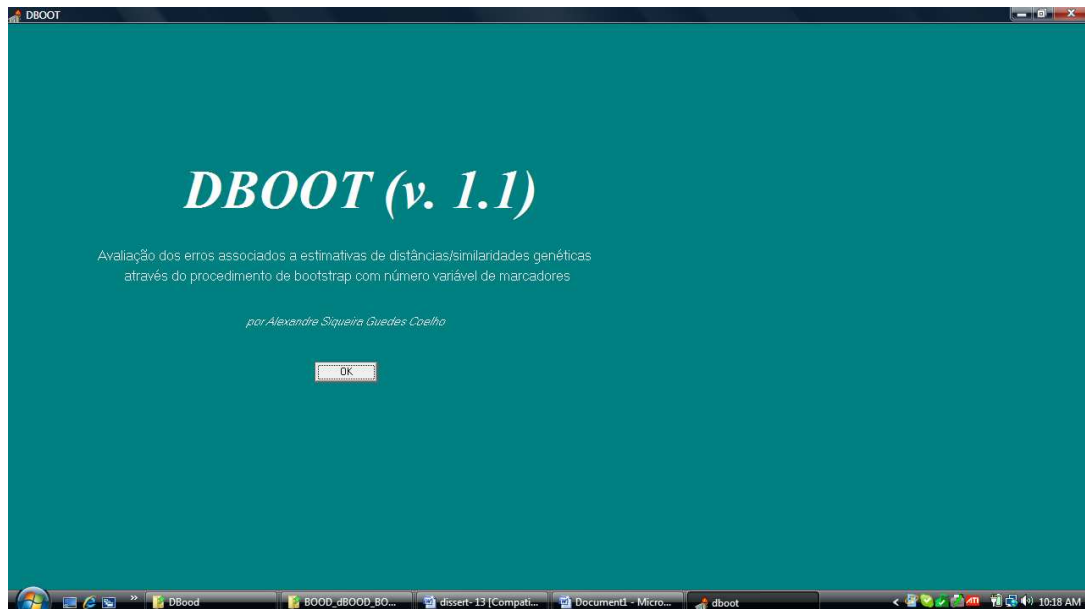
## **Appendix 8. DBOOT VERSION 1. 1**

### **I) About the program**

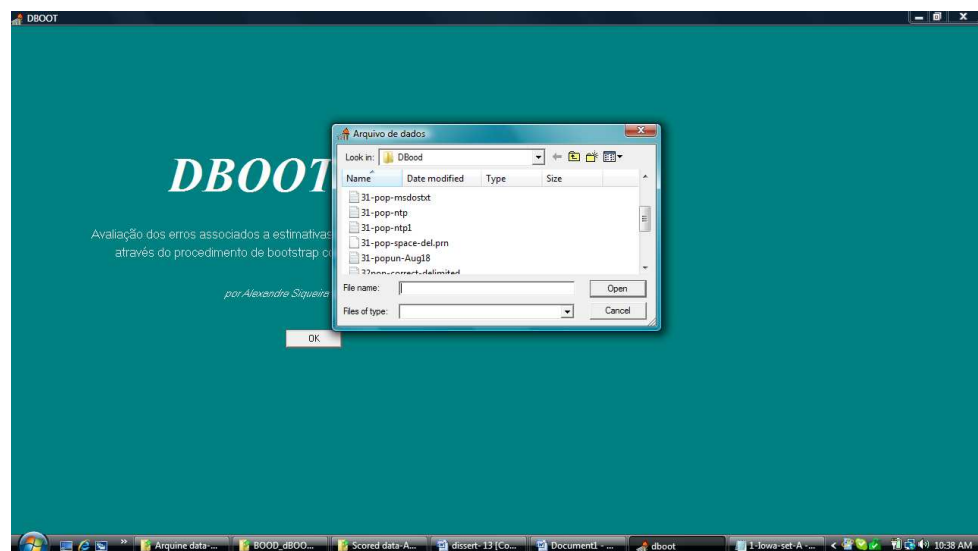
The program is designed to test the coefficient of variation for the molecular markers observed by assessing whether you have enough loci to explain the variation in your data set. The data set provided by the DBOOT program signifies the robustness of the data collected by comparing the coefficient of variation (percent variability) to the number of markers explored. The increasing number of markers used decreases the coefficient of variation at an increasing rate for a low number of markers. However, while continuing to add markers, the coefficient of variation decreases at a decreasing rate until it levels off to a point that adding more markers to assess variability is not needed. Without knowing the coefficient of variation for the number of markers observed, your results may not be as robust as they possibly could be by observing more markers. By observing more markers could possibly help explain more variation within the data you are assessing.

## II) Running the DBOOD program

- When you open the program you will see the following window.

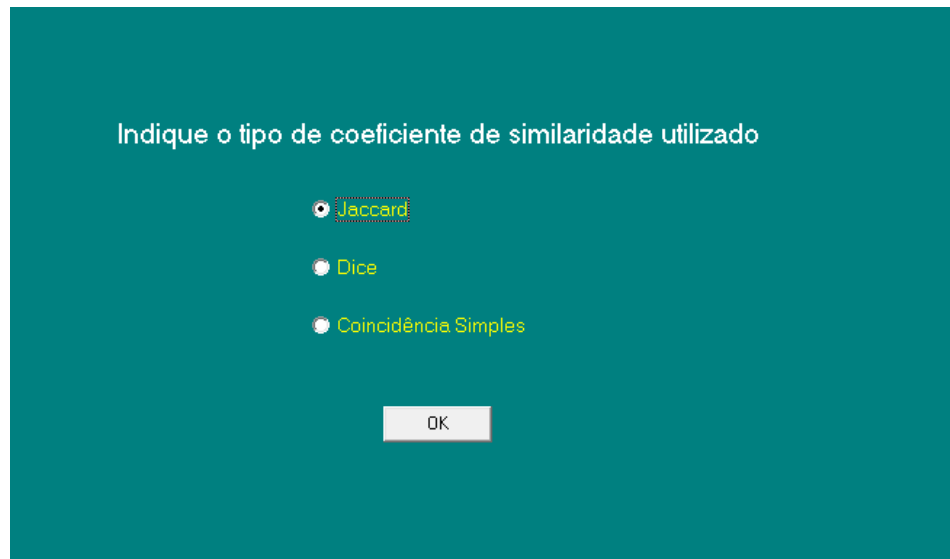


- You must click OK to begin once the program is started.
- You are then asked where the input file is located for importing into the program.





- The input file must have two characters at the top of the file to run properly and the name of the sample on a separate line to run properly. The first character (30) is the number of individuals (samples) for one location and the second character (221) is the number of loci observed. The numbers to the left represent the sample number (individuals).
- If there is a missing value, it must be in the form of a “?” . (Samples must be tab or fixed width delimited . To be tab delimited, it is much easier to use EXCEL and place each character in its own cell and save the file as a “Text Tab Delimited” file and open the file in NOTEPAD.
- **IMPORTANT:** Do not place any added spaces after the last loci for each sample as well as at the bottom of the file. The last line must be the last sample number.
- Please refer to the PDF file in Portuguese that is provided with the software if you do not understand the previous directions concerning proper spacing. If you do not space the data properly, the program will not run.
- After entering correct file for import you need to click Open.
- You will then be prompted to select the coefficient you wish to use for comparisons (i.e. Jaccard, Dice, or Simple Matching). In my case I have used the Jaccard coefficient.



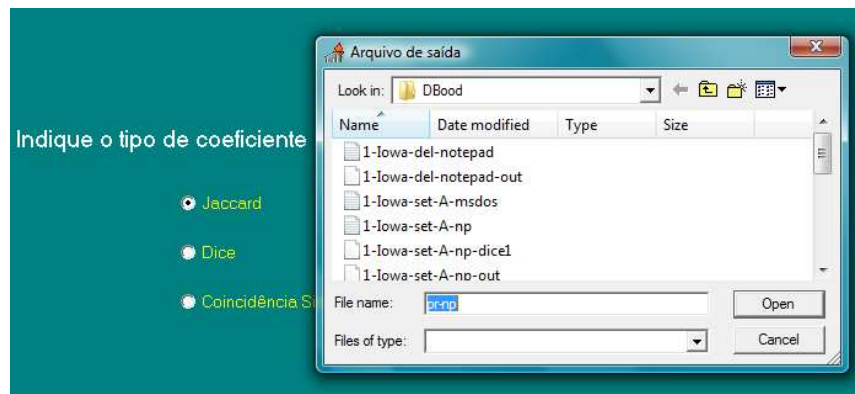
- After selecting the coefficient to use, click OK.
- The program will then ask you how many bootstrap iterations to perform.

It is beneficial to replicate 1000 times.

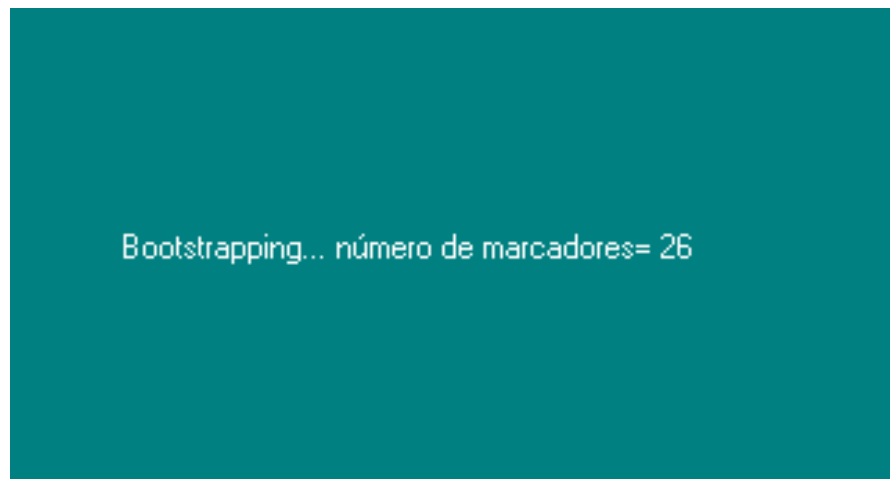


- After selecting the number of replications, click OK.
- The program will then ask you where it would like you to save the output file. Select a destination folder and name the file to be viewed in NOTEPAD. (The program will not save it in TXT format but you can open the file in NOTEPAD. Quite often you may be asked to select a

program from a list in order to open the program. At this time you may choose NOTEPAD.



- Once you have named a destination folder, click Open. The output file will then be saved to this destination you have now named.
- Once you click on open, you will see the following bootstrapping window.

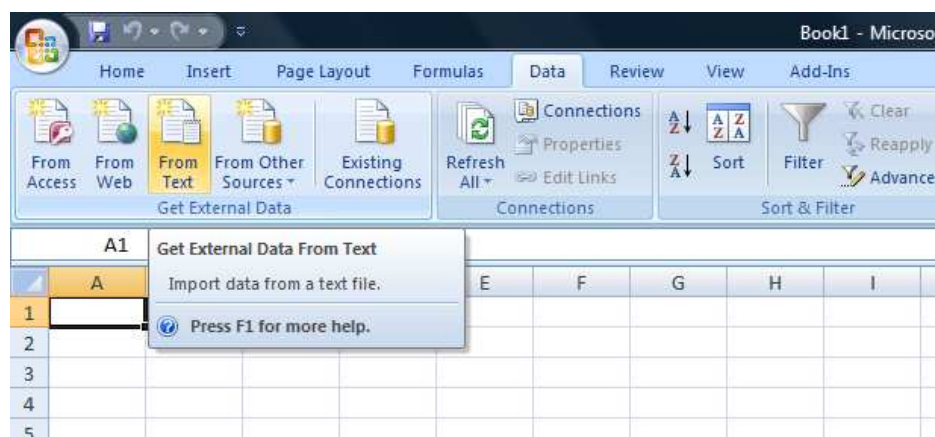




- Here example of is the DBOOD output in NOTEPAD.

no. bootstrp	no. locos	media	variancia	CV(%)	q(.25)	q(.75)
1000	1	0.698695	0.207710	65.819049	60.538145	70.852206
1000	2	0.699025	0.103285	46.375797	42.474076	50.111298
1000	3	0.696199	0.069775	38.269810	35.095233	41.321892
1000	4	0.694786	0.052316	33.207665	30.589615	35.749813
1000	5	0.698960	0.041189	29.278556	26.903520	31.571685
1000	6	0.695262	0.034787	27.055582	24.874494	29.226783
1000	7	0.695819	0.029708	24.987686	22.997548	26.976301
1000	8	0.696548	0.025864	23.289566	21.345046	25.130963
1000	9	0.695422	0.022976	21.983438	20.269544	23.852990
1000	10	0.695710	0.020819	20.918397	18.952929	22.758522
1000	11	0.696619	0.019318	20.126603	18.322835	21.711858
1000	12	0.696754	0.017343	19.065126	17.454412	20.567905
1000	13	0.696374	0.016007	18.327890	16.769053	19.742963
1000	14	0.695802	0.014824	17.648976	16.252149	18.911922
1000	15	0.694147	0.014183	17.309005	15.785548	18.724893
1000	16	0.697550	0.012952	16.450891	15.037912	17.644987
1000	17	0.698930	0.012334	16.022519	14.638285	17.308176
1000	18	0.696959	0.011541	15.544037	14.171754	16.791299
1000	19	0.698442	0.010774	14.985426	13.672956	16.165602
1000	20	0.697598	0.010494	14.810112	13.626992	15.953209
1000	21	0.698802	0.009821	14.303413	13.129676	15.399664
1000	22	0.697766	0.009352	13.979739	12.739518	15.087583

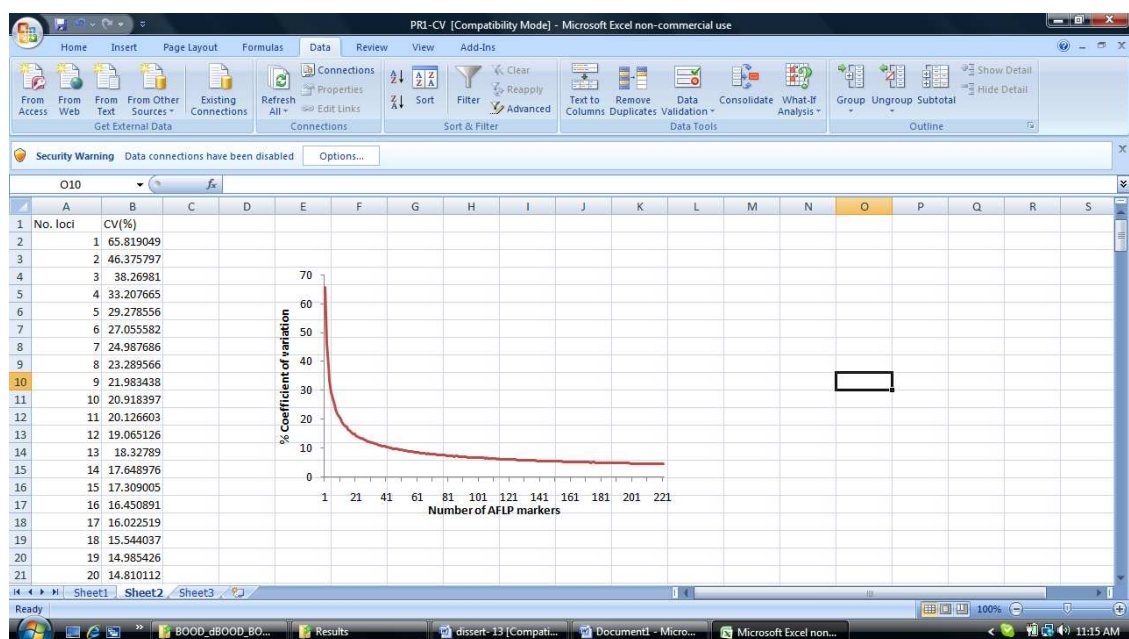
- To make the graph, open the DBOOT output in EXCEL. You will need to make sure the program will open the data allowing each column to be placed in separate cells so you can manipulate the data. Here example how you open it in EXCEL. You have to select- data-import from text.



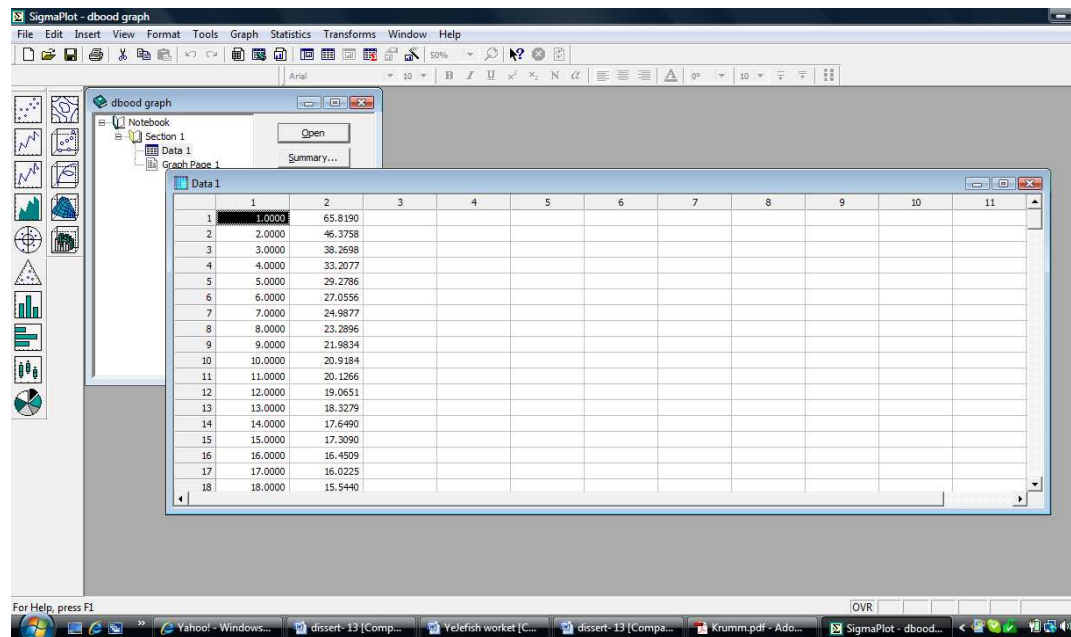
- Below is DBOOD output opened in EXCEL.

Security Warning Data connections have been disabled Options...								
H21								
	A	B	C	D	E	F	G	H
1	Coeficientes	de similarid	ade do tipo	Coincidencia	Simple			
2	no. bootstrp	no. locos	media	variancia	CV(%)	q(.25)	q(.75)	
3	723	1	0.628543	0.217755	78.333089	59.533419	95.072696	
4	895	2	0.631731	0.136596	61.217388	47.852249	72.591805	
5	976	3	0.636215	0.091008	49.589789	38.919149	58.832963	
6	993	4	0.635254	0.068962	43.099809	34.227948	50.555873	
7	1000	5	0.633056	0.053941	38.209729	30.227922	45.248628	
8	1000	6	0.632347	0.044788	34.923136	27.401074	41.497849	
9	1000	7	0.634305	0.038058	32.01744	25.439042	37.787818	
10	1000	8	0.635506	0.033248	29.871	23.550696	35.377047	

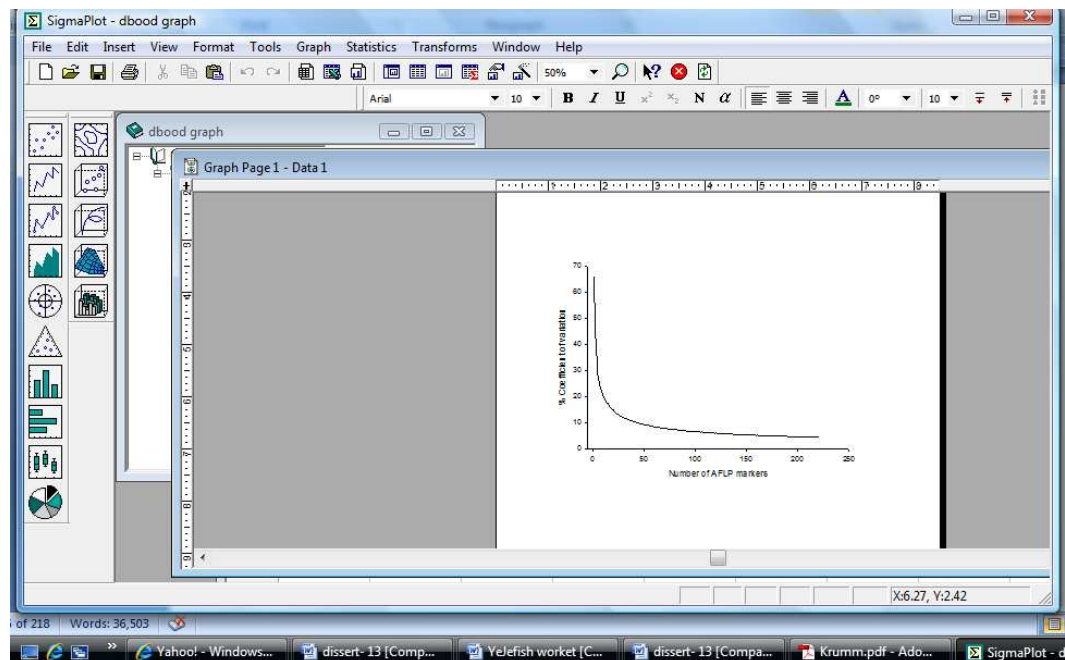
- Then you make the graph in EXCEL or SIGMA PLOT. Here is example of EXCEL graph.



- You can enter the data into SIGMA PLOT to get more quality graph.



- Below is a window showing a SIGMA PLOT line graph.



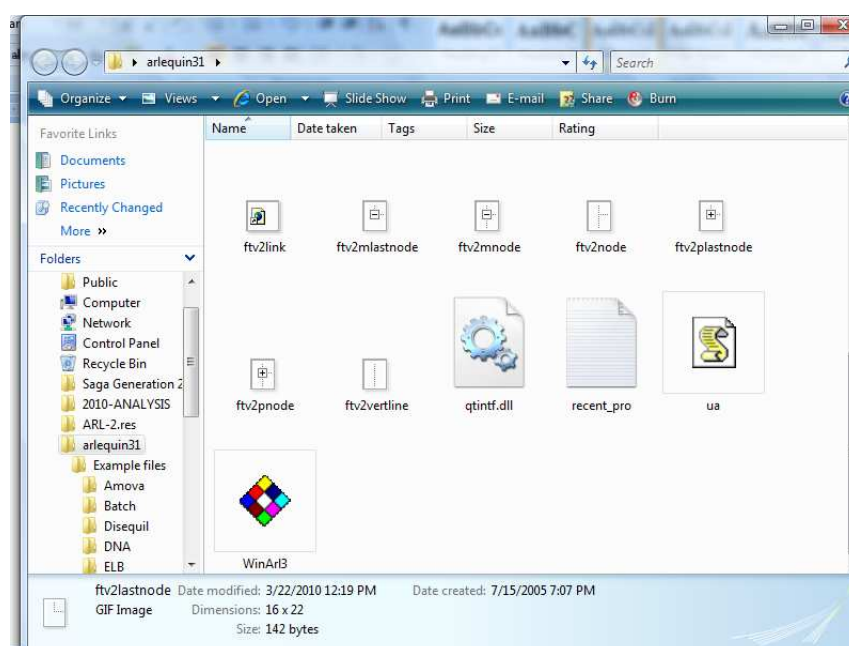
## Appendix 9. ARELEQUIN VERSION 3.1

### I) About the program

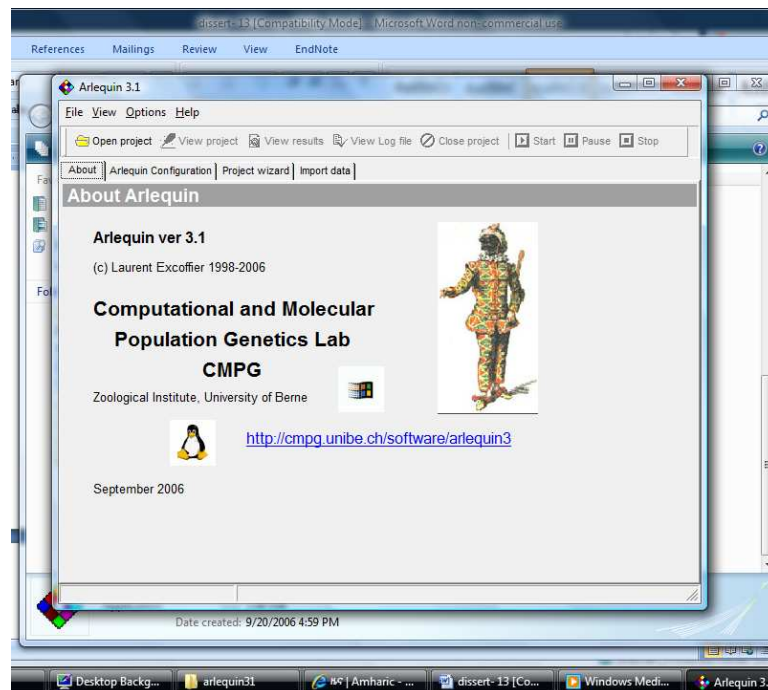
ARLEQUIN is a Windows-based computer package for the analysis of genetic diversity indices, analysis of molecular variance (AMOVA), computing minimum spanning trees, and exact tests of population differences. ARLEQUIN will accept data from DNA sequences, RFLP, AFLP, microsatellites, standard data, and allele frequency data. The main role of ARLEQUIN in the JEF laboratory is to analyze molecular variance by calculating  $F_{ST}$  ( $\Phi_{st}$ ). ARLEQUIN can be downloaded for free at the following website: <http://lgb.unige.ch/ARLEQUIN/>.

### II) Running the ARLEQUIN program

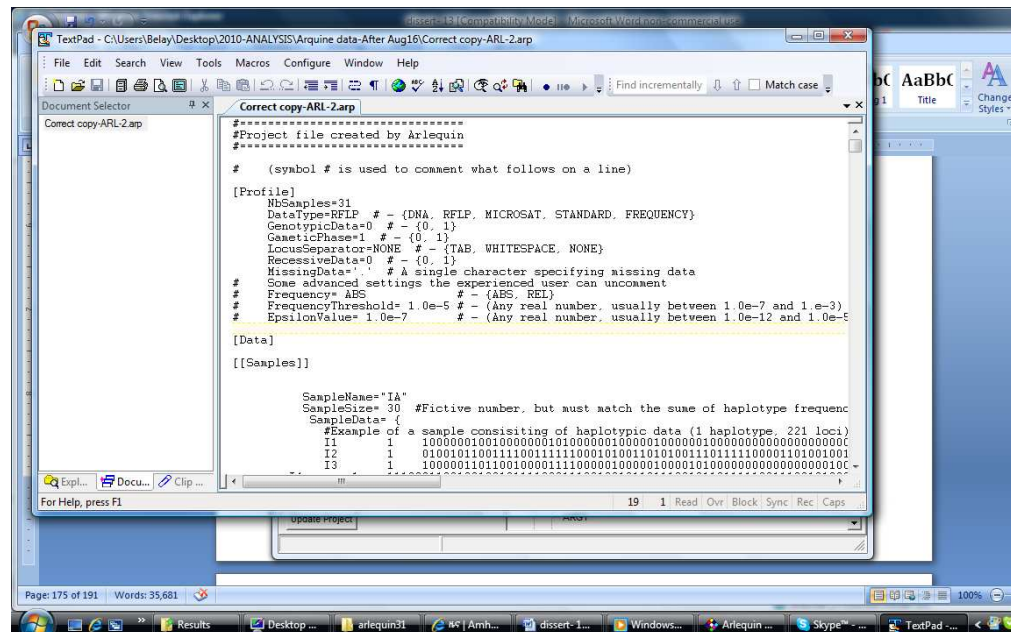
Below are the procedures and window screens which you may see during your ARLEQUIN experience. When you click on the ARLEQUIN folder, you find the following window. Double click on the diamond form icon to run the program



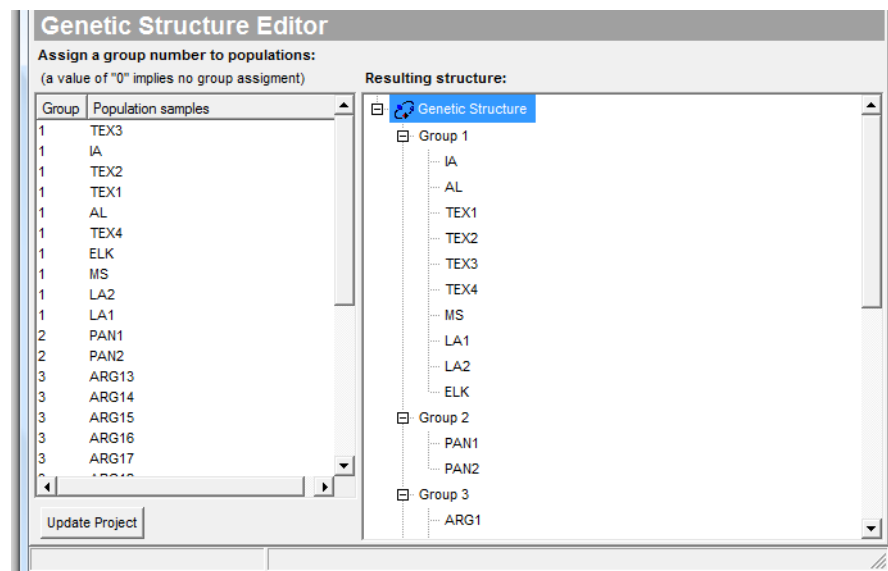
- The following window will open.



- The program is now running now and you must import data or go to project wizard to enter the data manually (copy and paste from TEXTPAD or NOTEPAD).
- To import data you should set it up into two files: a program file and a data file.
- The data file will be opened automatically from the external file command from the program file.
- Here is a window with an example program file:



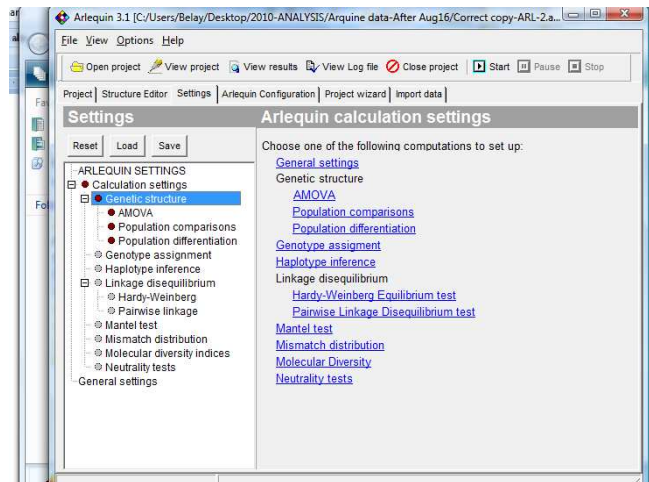
- Once the project is loaded, click on structure to group your samples. Below is grouping of my FAW samples.



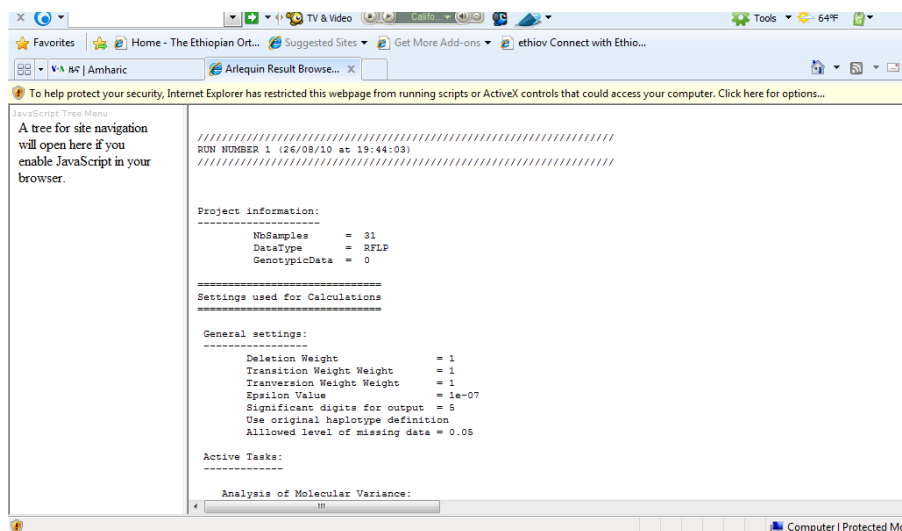
- Once the program is open, select Calculation Settings, then click on Genetic Structure, then click on AMOVA/ MSN and turn it on by checking the box.



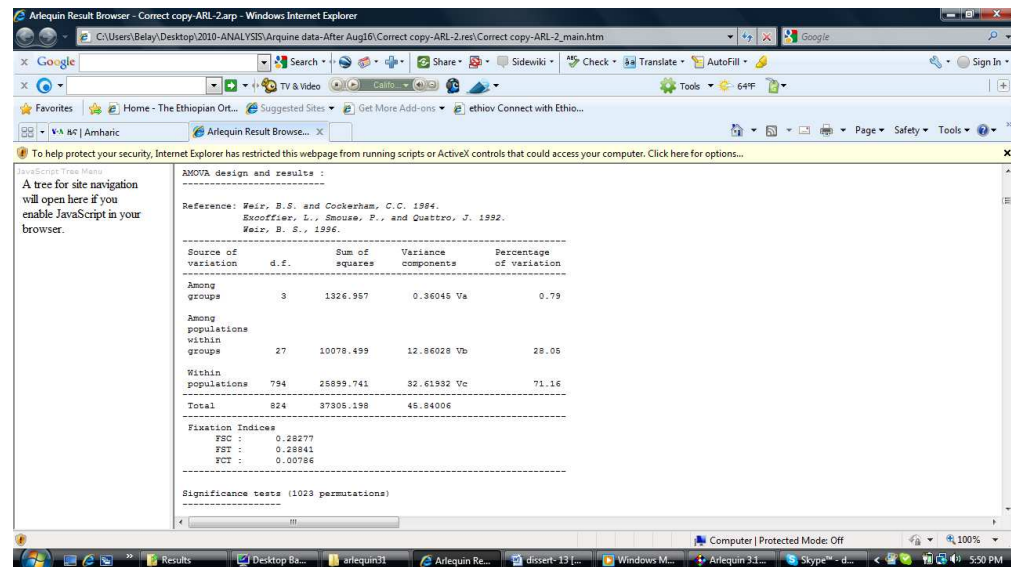
The “+” in front of the AMOVA/ MSN, means that it is turned on. Now run the program by clicking the “start” button.



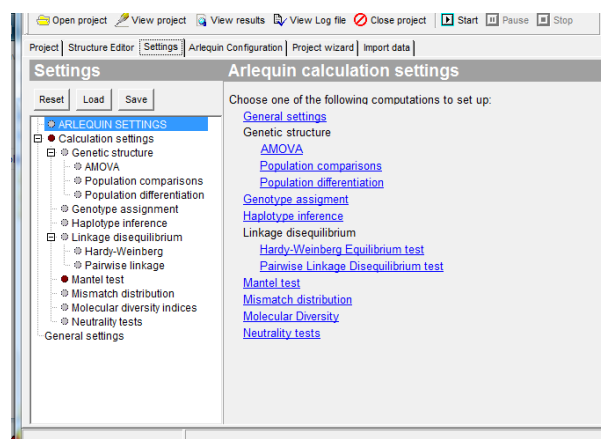
- Below is the first information you will see in the output window. Scroll down to get AMOVA table and other information.



- Below is the AMOVA table

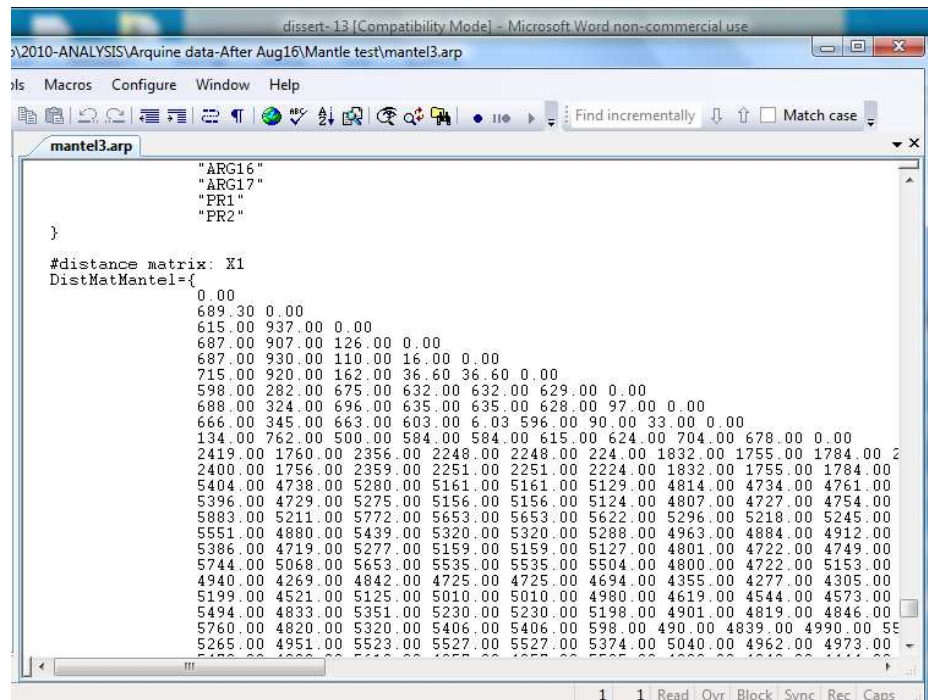


- If you want to know the correlation between genetic and geographic distance, run Mantel test.

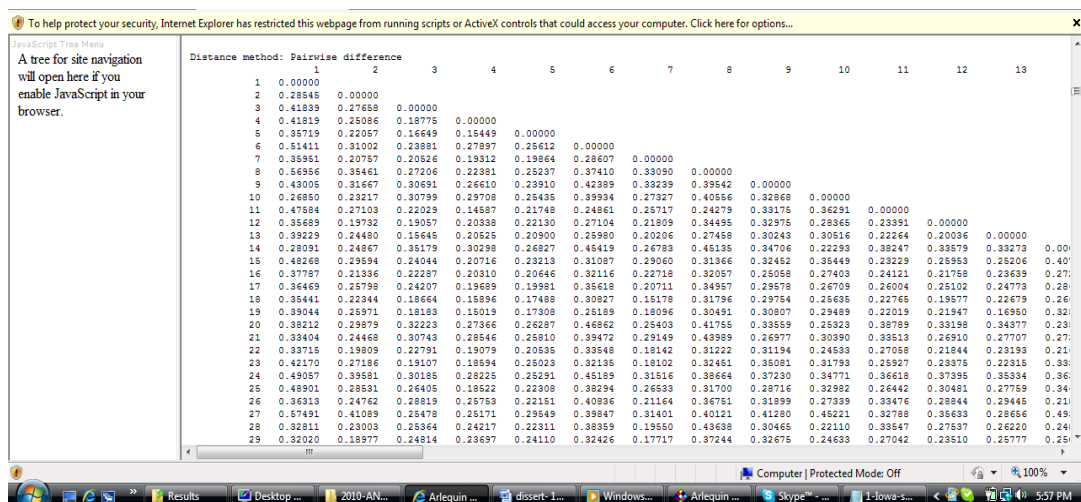




- To run the Mantel test, you need the geographic and genetic distance matrixes. For the genetic distance either you can use the ARLEQUIN output directly or enter it manually. Here is example of geographic distance matrix entered into ARLEQUIN.



- Example of genetic distance matrix in ARLEQUIN.



- Mantel test output example.
- The data will be output into a folder with a .res extension.

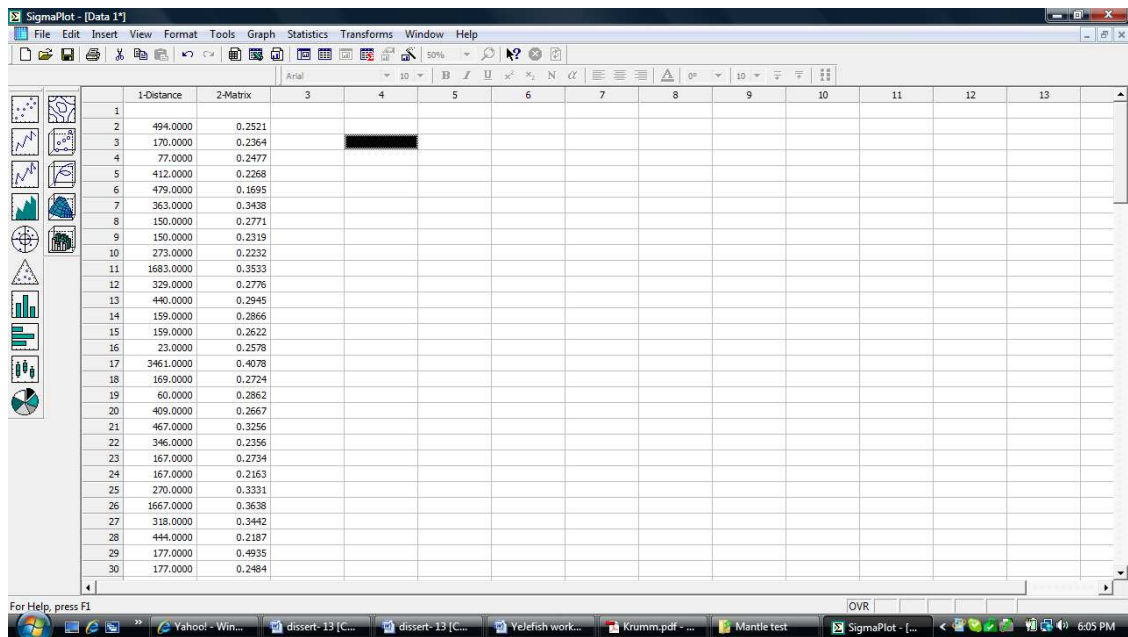
To help protect your security, Internet Explorer has restricted this webpage from running scripts or ActiveX controls that could access

JavaScript Tree Menu  
A tree for site navigation will open here if you enable JavaScript in your browser.

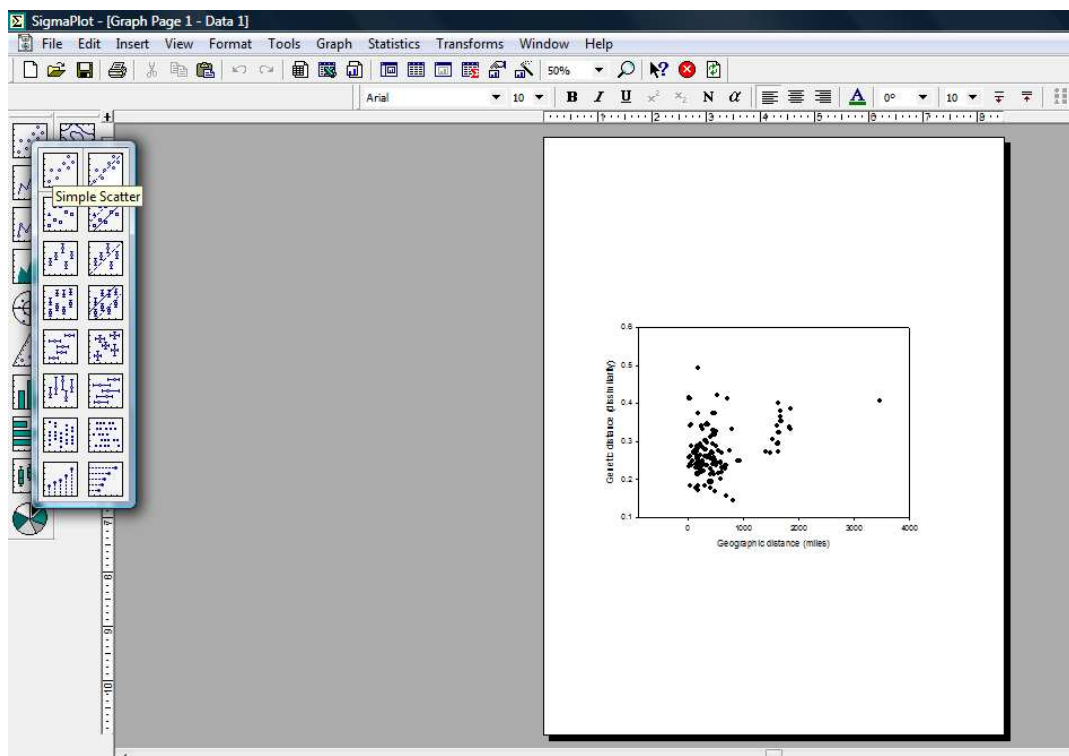
Mean value Y	: 0.284281
Sums of squares Y	: 2.363190
Mean value X1	: 2690.068989
Sums of squares X1	: 1995308971.562823
ZY1	: 362560.487007
Sum of products (SP(Y,X1))	: 6960.343324
Regression coefficient (bY1)	: 0.000003
Correlation coefficient (rY1)	: 0.101362
Determination of Y by X1(%)	: 0.010274
Significance testing (1000 permutations for Mantel test)	
=====	
P(rY1 rand >= rY1 obs)	: 0.030000
No. of smaller rand rY1	: 970
No. of equal rand rY1	: 0
No. of larger rand rY1	: 30
//	
END OF RUN NUMBER 1 (27/08/10 at 12:30:00)	
Total computing time for this run : 0h 0m 7s 394 ms	
//	

- The output will open up in Windows Explorer. You can get the correlation and regression coefficients and their probability values from the above output.
- You can make the isolation by distance graphs in EXCEL or SIGMA PLOT. Below is example of SIGMA PLOT file input and graph.

- Below is SIGMA PLOT data input window.



- Isolation by distance scatter plot graph in SIGMA PLOT.



## **Appendix 10. POPGENE VERSION 1.32**

### **I) Introduction**

POPGENE is a Windows-based computer package for the analysis of genetic variation among and within natural populations using co-dominant and dominant markers (i.e. AFLP, RAPD, and RFLP) using haploid and diploid data sets. It can be used to create summary statistics such as allele frequencies, gene diversity (heterozygosity), genetic distance, and F-statistics for (1) single-locus, single populations; (2) single-locus, multiple populations; (3) multi-locus, single populations and (4) multi-locus, multiple populations.

### **II) Installation Instructions**

POPGENE can be downloaded for free at the following website:

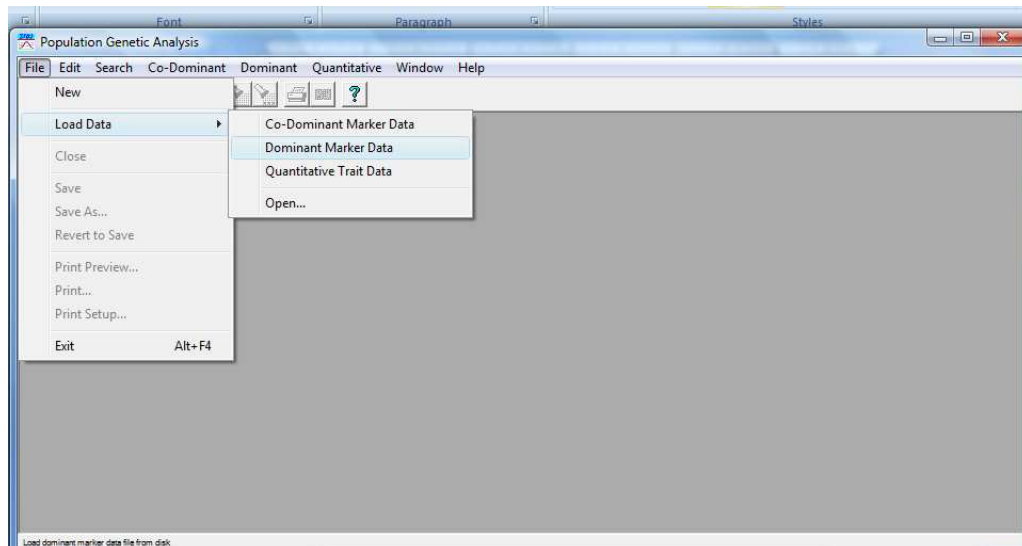
<http://www.ualberta.ca/~fyeh/download.htm>

The following are the steps required for POPGENE installation.

1. Create a sub-directory on your computers hard drive and label it POPGENE.
2. You can now visit the website and download POPGENE into the directory that you have just created. The website will give you additional directions and a user's manual if you wish to download.
1. Double click on the newly saved file and follow the directions on the computer screen. It is at this point you will be asked where to save the POPGENE.EXE file.
2. You can now locate the POPGENE icon and double click to start running the program.

### **II) Running the POPGENE program**

Below are the procedures and window screens which you may see during your POPGENE experience.



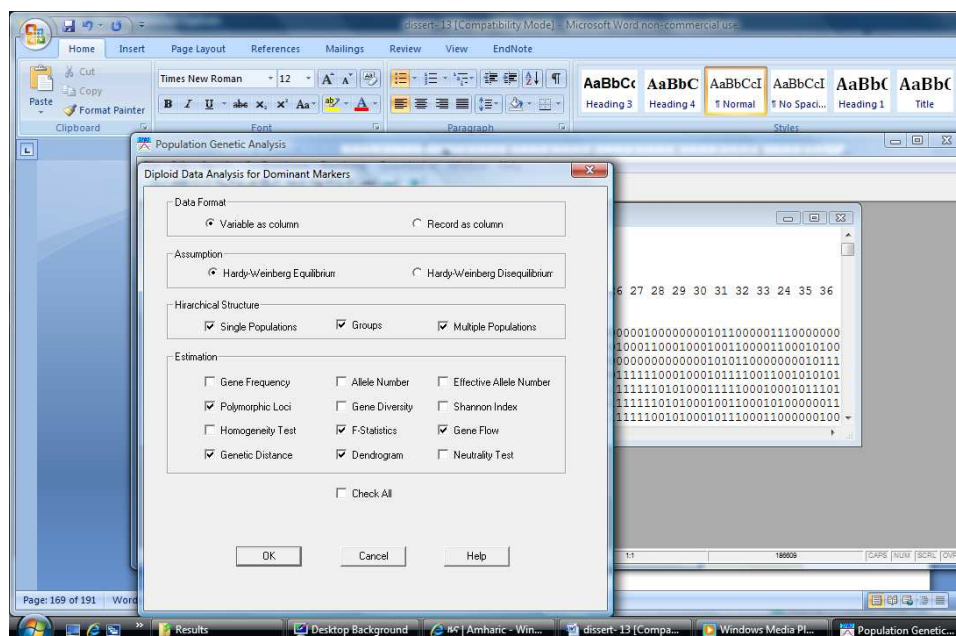
- From the file menu, click on “Load Data”
- You then must choose the data type you wish to load. In many cases it may be AFLP “Dominant Marker Data”. You will then need to specify the location of your input file (which must be in the form of a .TXT file).
- Your file must contain the following input parameters:
  - /\*FAW Population Analysis\*/ (this is the output name given)
  - Number of populations = 31 (self explanatory)
  - number of loci = 221 (number of markers observed)
  - Locus name : (you may name the markers, in my case I named them with numbers corresponding to the number of markers analyzed)
  - Population name (name = population name)



**Gene Flow:** Estimates gene flow from the estimate of  $G_{ST}$  or  $F_{ST}$ . This estimation is also made for Groups and Multiple Populations.

**NOTE:** The above mentioned modules was used for the analysis of FAW for this dissertation. However, there are many more modules that can be used such as, Genetic Distance, and the respective Dendrogram both available for Groups or Multiple Populations. However these modules will not be discussed but can be addressed in the POPGENE “help” file or the users manual.

## V) User Interface Windows

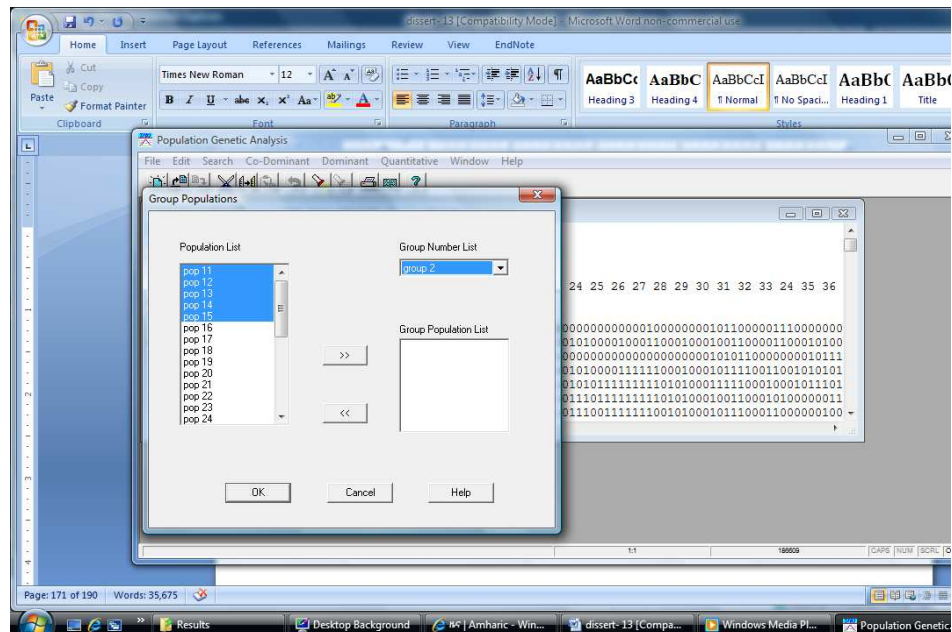


- It is at this window that you must decide which modules you wish to run. If you are unsure which modules to run, click “check all” and all modules will be assessed. You may choose to look at each population individually. However, if you are looking at Multiple Populations, you have the option of choosing if you would like to analyze a “group” or region of populations. For example: you have populations from United states, Argentina, Panama, and

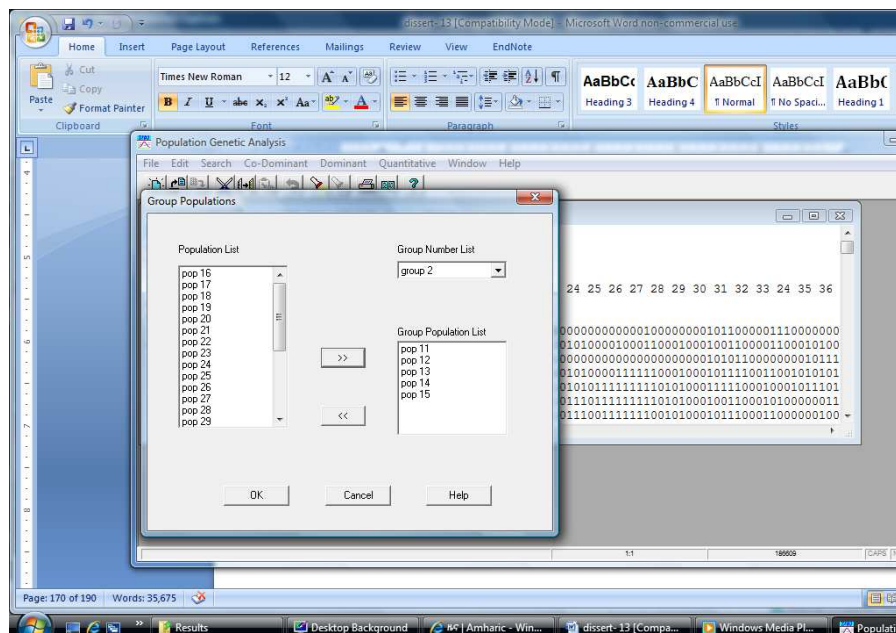
Puerto rico, you may choose to “group” samples from those locations together essentially in four groups. This analysis is similar to what AMOVA requires to run its analysis. This analysis can be accomplished by clicking the “Single” “Groups”, or “Multiple Populations” under the “Hierarchical Structure” heading as seen in the previous window.

- There will be a series of two query windows that will appear. The first will ask if you care to retain all loci for further analysis, so click “yes”. The next window will ask if you care to retain all populations for further analysis, so click “yes”. By clicking NO, you will be asked to delete those populations and loci from further analysis. By doing so you will bias your data by deleting loci and populations.
- The next window will then ask you the number of groups to choose. In the previous example I chose 4 groups.
- Click OK after selecting the number of groups.
- The following window then asked to specify groups that you had previously determined.





- By highlighting particular populations enables you to group them in the “corresponding group number”. By clicking the arrow button moves the selected populations to the “Group Populations List” as shown in the window below.





## **Appendix 11. NT-SYSPC -VERSION 2.11 program**

### **I) About the program**

The NTSYS software was designed to display structure in multivariate (numerical) data. For example, you may want to find out where or how individuals are related. Methods furnished in NTSYS are largely associated with the field of phenetics, however, Satiou and Nei's neighbor-joining methods of phylogenetic tree estimation is included in this software package.

Most of the data that you will use to be inserted into NTSYS will be in the form of a data matrix consisting of 1's and 0's, however, many types of data can be analyzed with this software. You will not find a lot of information in the user's manual about how and when to use certain procedures. The authors assume you must know a little about the field of statistical analysis of binary data. However, you will find the users manual helpful (which can be found in the data package by clicking on "help") for setting up data matrixes the appropriate way. Keep in mind that OTU's represent the number of samples and terms such as "variable" or "characters" refer to the number of loci you are analyzing.

### **II) Program Modules in NTSYS**

**MXCOMP** – This module compares to symmetric matrices by computing their correlation and plots a scatter diagram in the form of a Mantel test. Such as looking at the relationship of Geographic Distance with Genetic Distance or Similarity.

**SAHN** – Performs sequential agglomerative, hierarchical, and nested clustering methods. The most commonly used method is the UPGMA method which can be chosen while the program is running. If and when ties are found when computing matrices (can be very

common when looking at very large data sets), the program can find alternatives and builds different trees when running the “FIND” instead of the “WARN” option (which will be described later).

**SIMQUAL** – Computes various similarity or distance matrices for qualitative data (such as 1’s and 0’s). It is in this stage you assign the coefficient of choice such as simple matching (SM) Jaccard, Dice, etc.

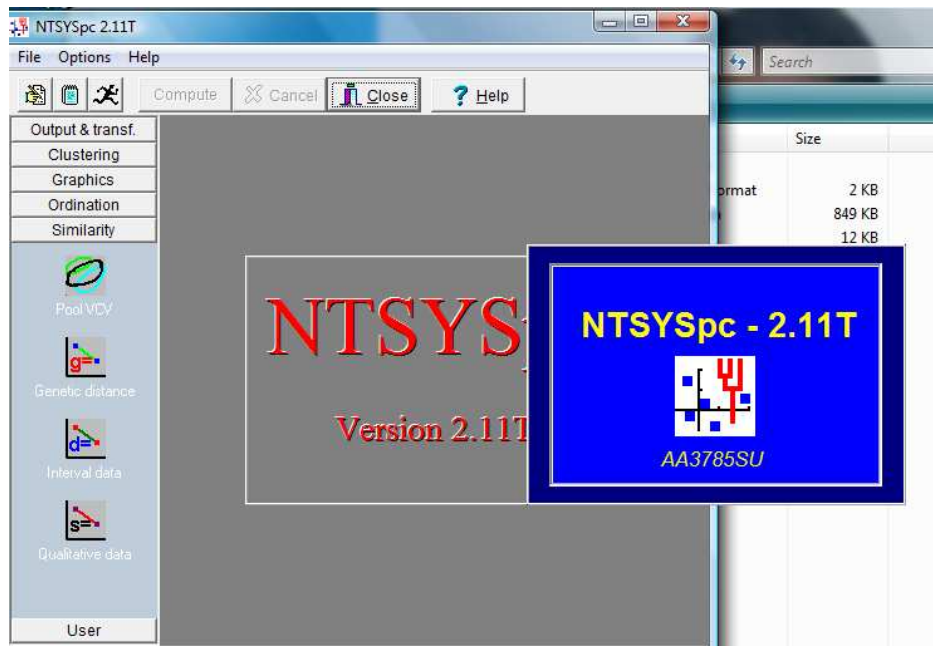
**TREE** – Displays a tree from your cluster analysis as a dendrogram. Keep in mind there is an option button at the top of the tree window that allows the manipulation of scaling, character fonts, and line fonts.

## **II) Getting Started Using NTSYS**

You will need to make sure you have the program installed on your computer. If you need to install the program, you will need the KEY CODE in which the software is licensed to JEF labs. When running NTSYS the first time you will need this KEY CODE and REGISTRATION number. Please keep these documents in a safe place for future use. Before running the program, you need to delimit and open the data file in Excel format.

- The window below is the first window that will open while running NTSYS.

It is here you will have the ability to pick and choose all the modules to run you wish.



- Notice the help menu and the top of the window. You may find this useful.
- Here is the format of EXCEL for NTSYS.

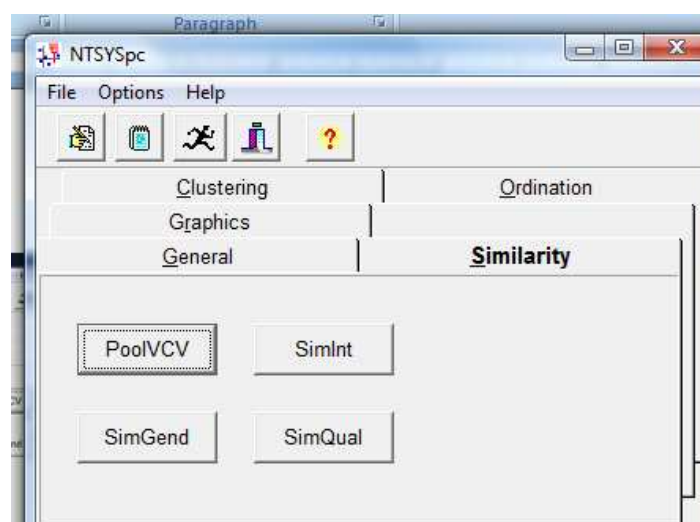
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	1	155	221	1	99														
2		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
3	IA1	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
4	IA2	0	1	0	0	1	0	1	1	0	0	1	1	1	1	0	0	1	1
5	IA3	1	0	0	0	0	0	1	1	0	1	1	0	0	1	0	0	0	0
6	IA4	1	1	1	0	0	0	1	1	0	0	1	0	0	1	0	0	1	0
7	IA5	1	0	0	0	0	0	0	1	0	1	1	1	1	0	1	0	0	0
8	AL1	1	1	0	0	1	1	0	0	1	1	1	1	1	1	0	0	0	1
9	AL2	0	0	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1
10	AL3	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1
11	AL4	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	0	0	1
12	AL5	0	0	0	1	1	1	0	1	1	1	1	0	1	0	1	0	0	1
13	TX1-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	TX1-2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
15	TX1-3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
16	TX1-4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	TX1-5	1	1	1	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0
18	TX2-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
19	TX2-2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
20	TX2-3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
21	TX2-4	0	0	0	0	1	0	0	0	1	1	1	1	0	0	1	1	1	1
22	TX2-5	1	0	1	1	1	1	1	1	1	1	1	0	1	0	1	1	0	1
23	TX3-1	1	0	0	0	1	0	0	1	0	1	1	1	1	0	1	1	0	1

- The file must be set up accordingly. It is easier to import the 1's and 0's matrix from EXCEL using Tab-delimited text. A number 1 must go in the first space followed by the number of individuals or samples (155) followed by the number of loci observed (221). A number 1 should appear next if you have missing data. However, if there is no missing data, this value should be a zero. If there is missing data, the next value should signify what the missing data is. In this case it is a "99". **NOTE:** You should also put an L AFTER the input number if the number represents something other than numbers such as sample names. For example the input statement should look like this if you use letters to signify individuals or samples (i.e. 1 155L 221 1 99).

- The flowing is a data matrix imported in to NTSYS program from EXCEL.

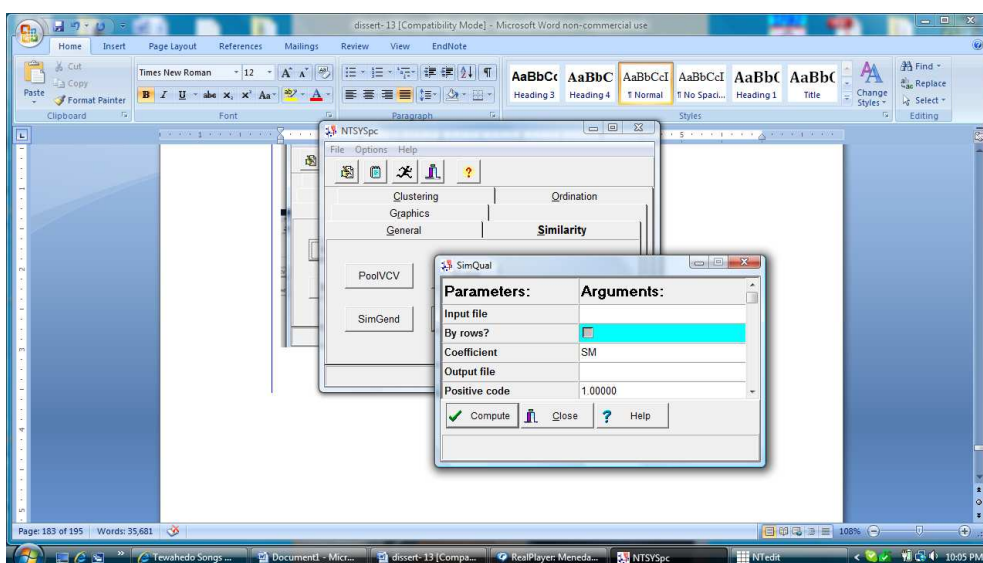
Rows\Col	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
IA1	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
IA2	0	1	0	0	1	0	1	1	0	0	1	1	1	1	0	0	1	1
IA3	1	0	0	0	0	0	1	1	0	1	1	0	0	1	0	0	0	0
IA4	1	1	1	0	0	0	1	1	0	0	1	0	0	1	0	0	1	0
IA5	1	0	0	0	0	0	0	1	0	1	1	0	0	1	0	0	0	0
AL1	1	1	0	0	1	1	0	0	1	1	1	1	1	1	0	0	0	1
AL2	0	0	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1
AL3	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1
AL4	1	1	1	1	1	1	1	1	1	1	0	0	1	0	1	0	0	1
AL5	0	0	0	1	1	1	0	1	1	1	1	0	1	0	1	0	0	1
TX1-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TX1-2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
TX1-3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
TX1-4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TX1-5	1	1	1	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0
TX2-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
TX2-2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
TX2-3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
TX2-4	0	0	0	0	1	0	0	0	1	1	1	1	0	0	1	1	1	1
TX2-5	1	0	1	1	1	1	1	1	1	1	1	0	1	0	1	1	0	1

- If you are entering binary data (such as 1's and 0's), you will first need to construct a similarity or a distance matrix (in the form of a triangle) by selecting the “Similarity” button.



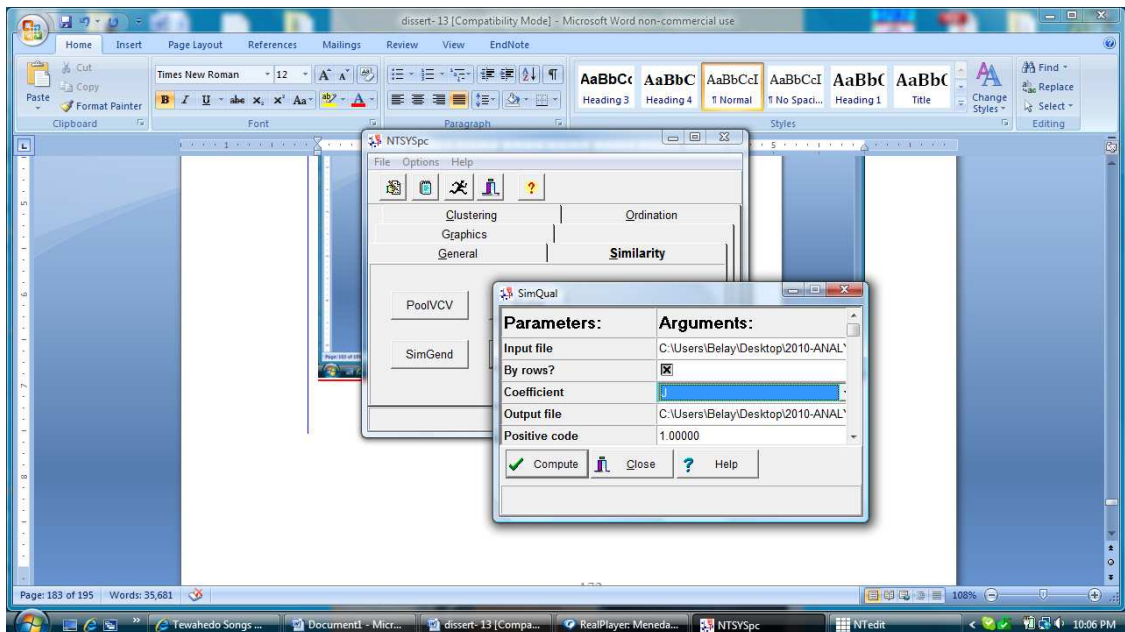
- After clicking on the “similarity” button use the SIMQUAL module.

- Here in this window you will need to import your 1 and 0 matrix into the “input file” column. **NOTE:** You can click on the white space above to create drop down windows that would not appear otherwise. You then can choose your coefficient and name an output file to save your similarity matrix to.

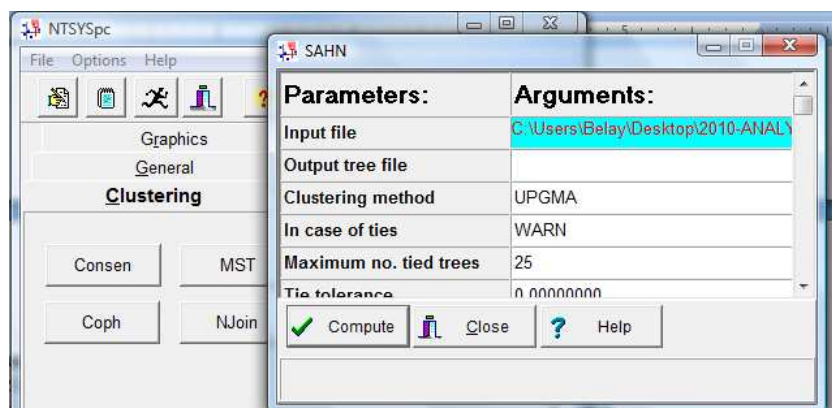


- If you are setting up your input file where the samples are in columns, you do NOT check the box specifying “by rows?”. However, if your data is in rows you need to check the box.
- Below is a sample input file you can use to set up your NTSYS file.



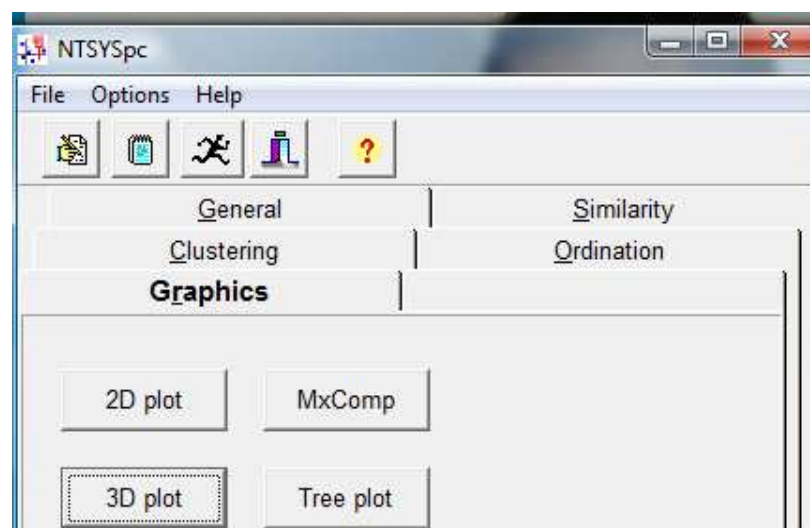


- Once you have named the output file accordingly and specified a correct input file, you may click on “compute”. The file will run and you will now have a similarity matrix that you can perform cluster analysis with.

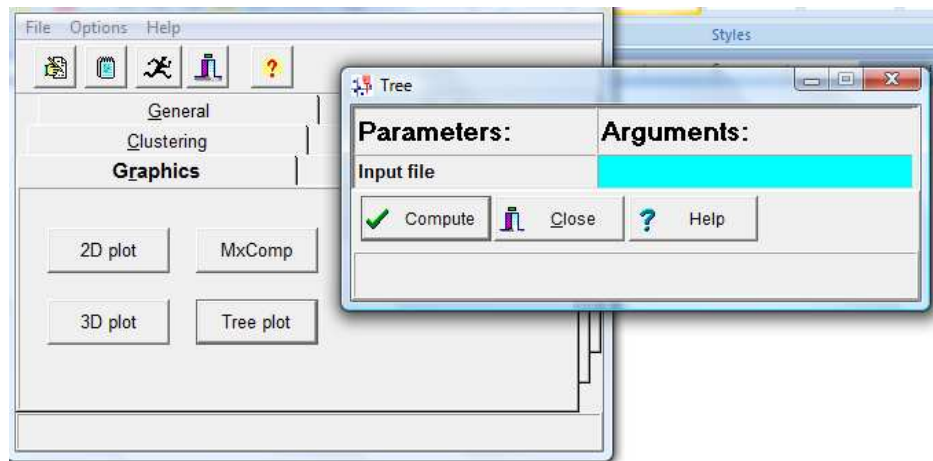


- You are now ready to perform cluster analysis using the SAHN module.
- Below is a window that will appear by clicking on “clustering”.
- You will now want to click on “SAHN” to perform cluster analysis.

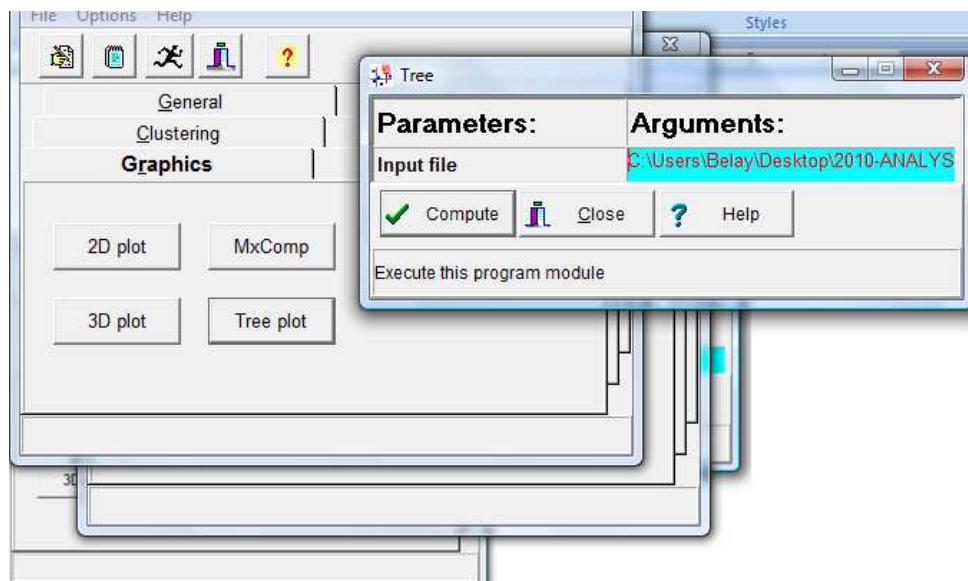
- You then will need to type or browse for the file by clicking in the white area specified as “input file” that you had previously saved (the similarity matrix produced by NTSYS using SIMQUAL) and place it in the “input file” box.
- You will then need to name the output tree file.
- You can also change the type of clustering method. However, in most cases you will probably choose to use the UPGMA method.
- Once the output tree file has been named, you will then need to change the default settings that “warn” of tied trees to “find” by clicking on “warn”. This is due to the possibility of tied trees (if you have a large data set you probably will have a couple tied trees). If you have a large amount of tied trees you may need to change the number of tied trees from 25 to a larger number.
- You then can click “compute” to generate a tree plot file.
- Then click on graphics to produce the dendrogram and the following window will appear.



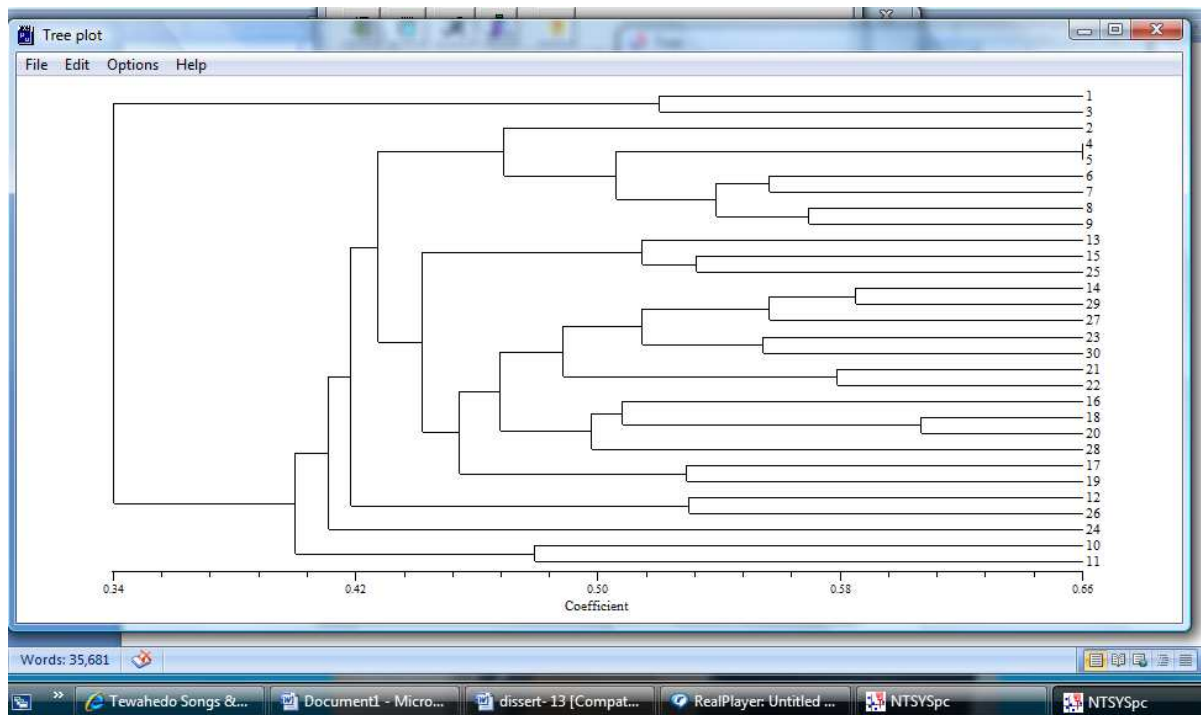
- Now you will be asked for the input file which is the previous output.



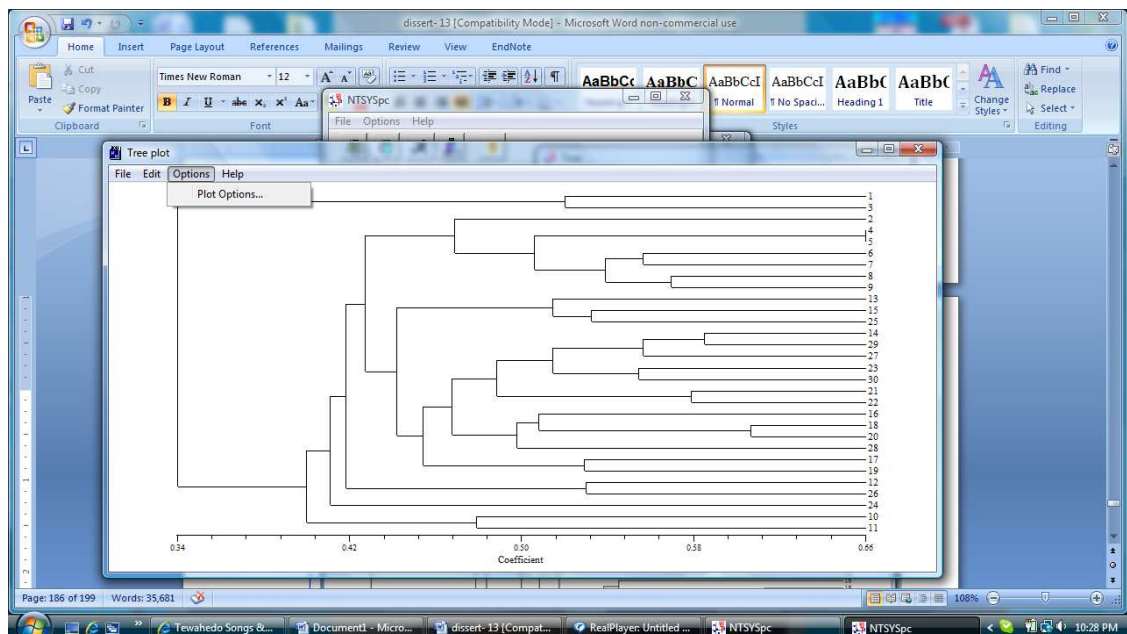
- The browse the input file and press “compute”.



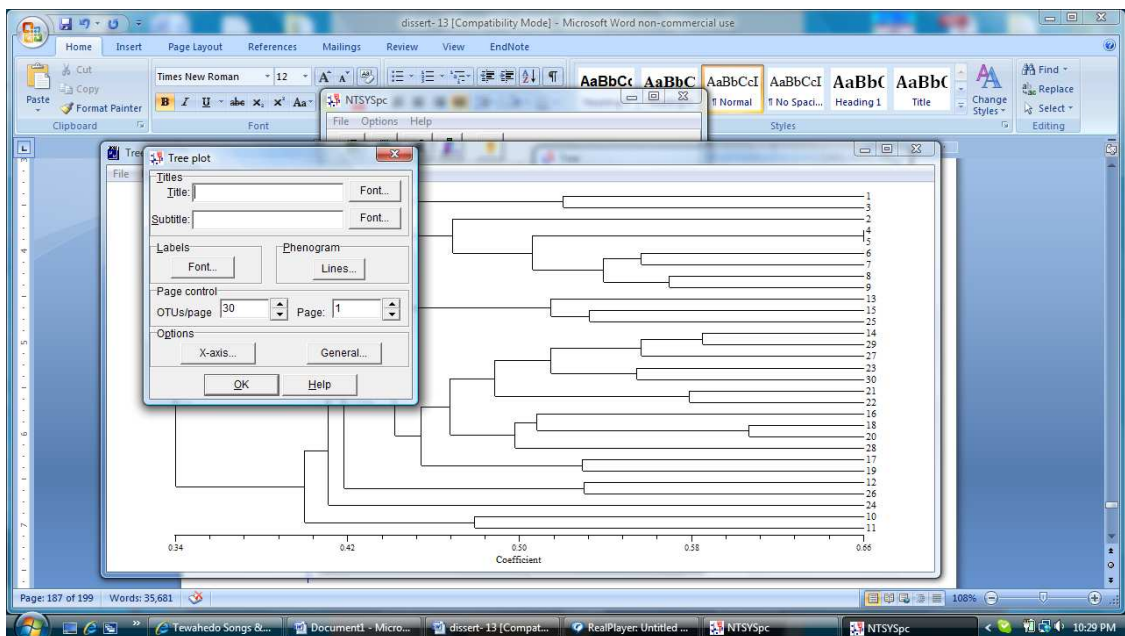
- At this step the dendrogram will appear as seen below.



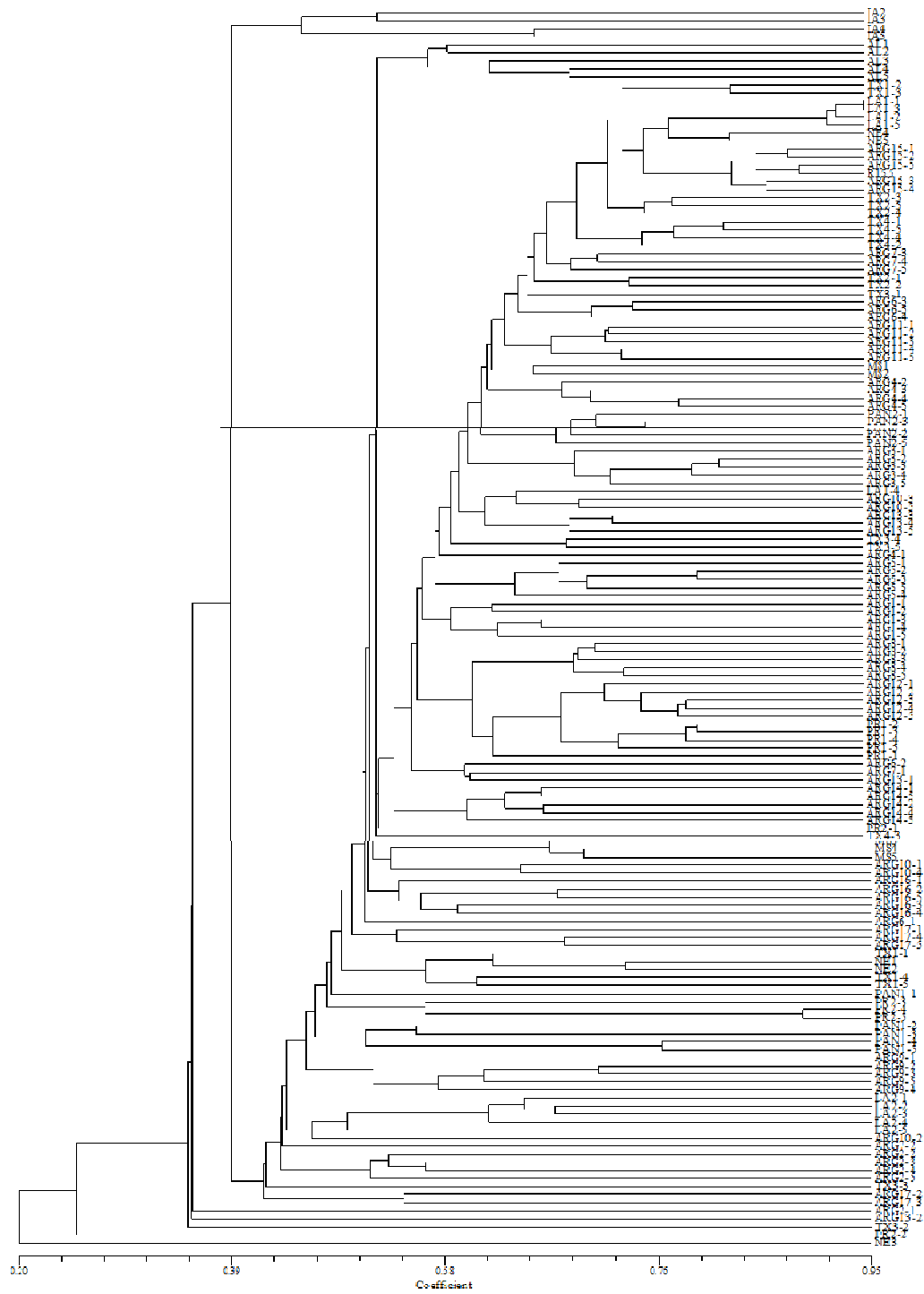
- Now you can play around to improve your graph as you need using the option menu as shown here.



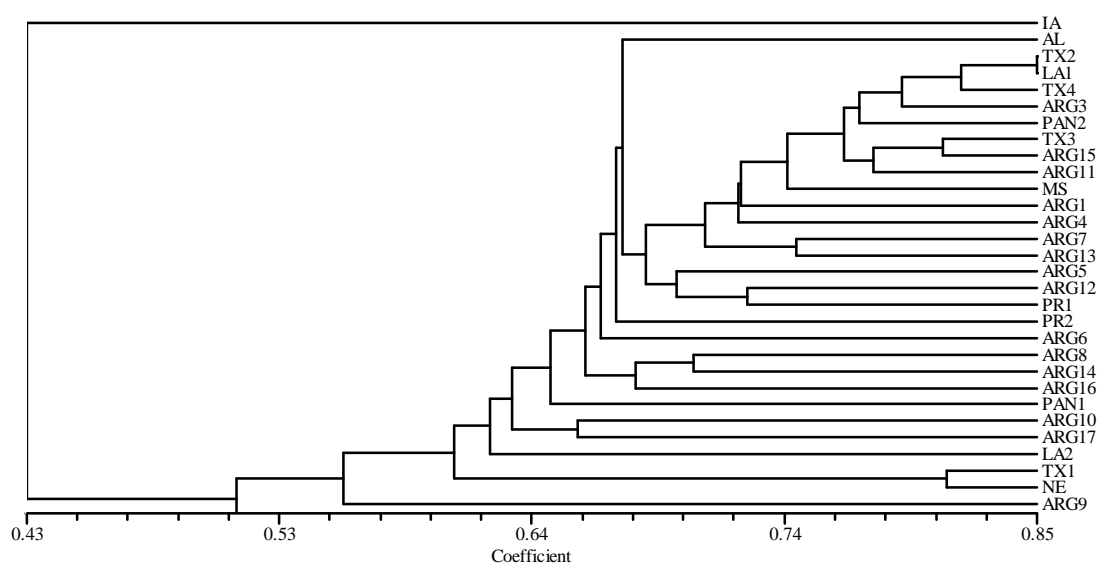
- In the option menu, a small new window will open that allows you to edit title, fonts, line thickness, etc.



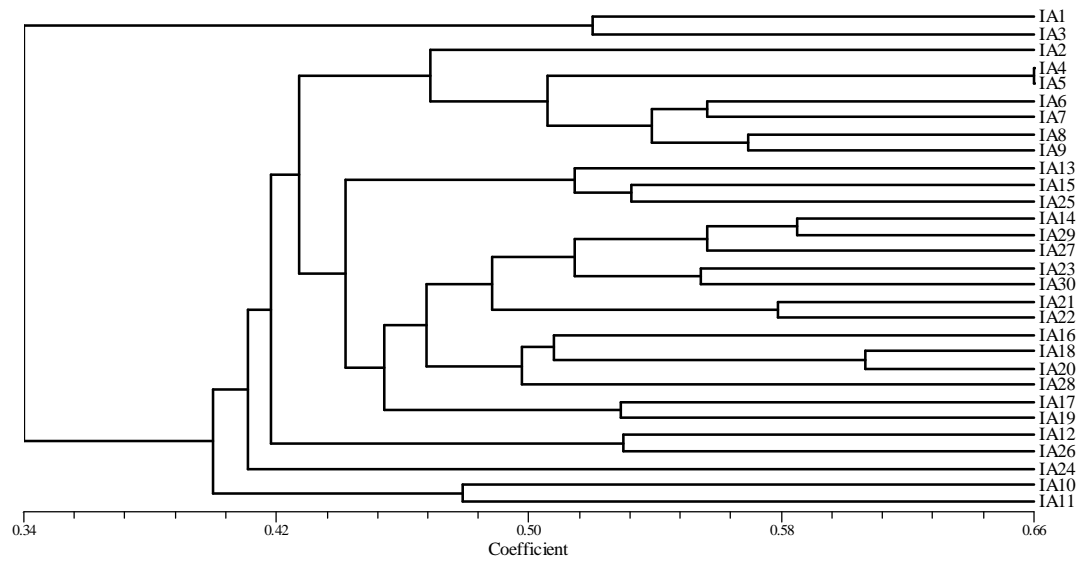
- Now go to edit- copy meta file-and then you can paste the dendrogram into your word document. Below are examples of graphs produced following the above procedures of NTSYS for different FAW populations used in the present study.



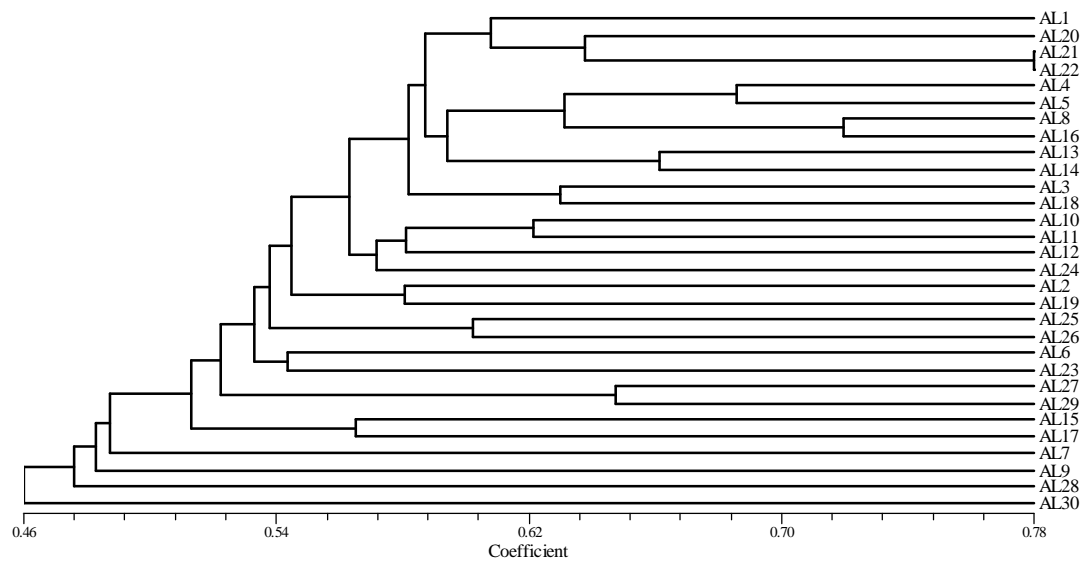
**Figure 32.** Dendrogram of 5 samples from each 31 FAW populations used in the study.



**Figure 33.** Dendrogram of one individual sample from each 31 FAW populations used in the study.

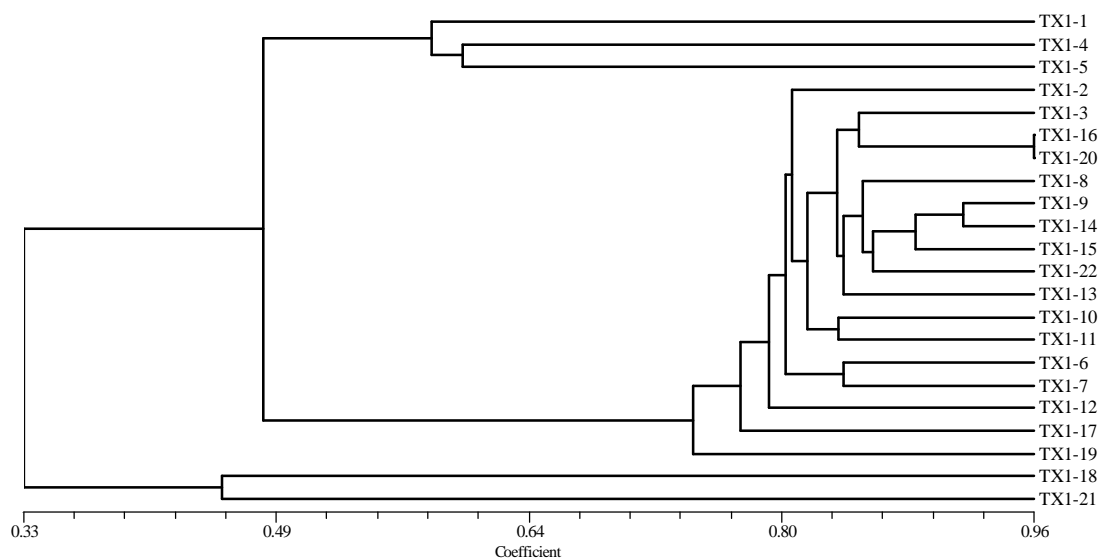


**Figure 34.** Dendrogram showing the interpopulation relationships of Iowa FAW sample.

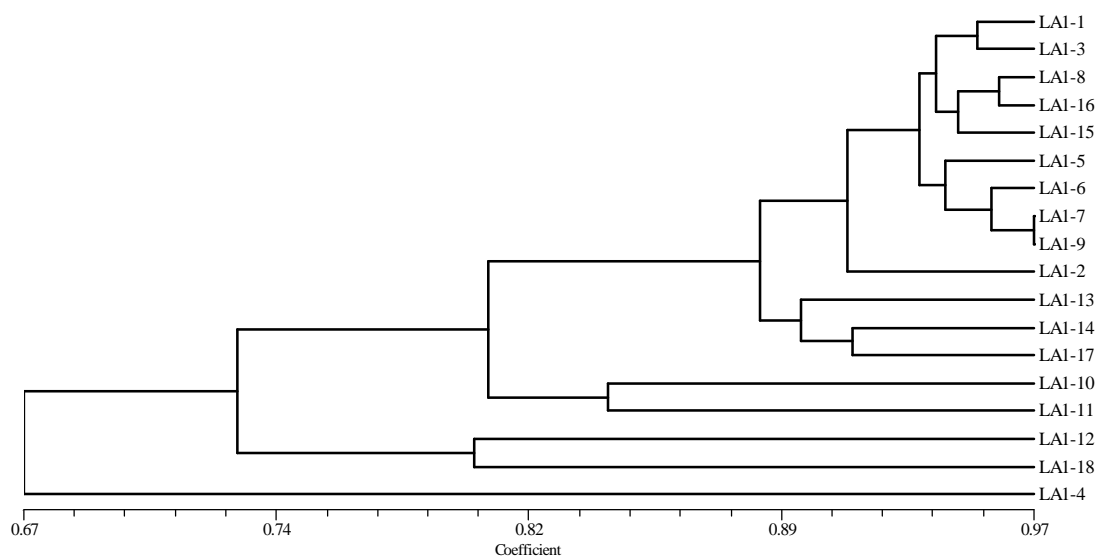


**Figure 35.** Dendrogram showing the interpopulation relationships of Alabama FAW population.

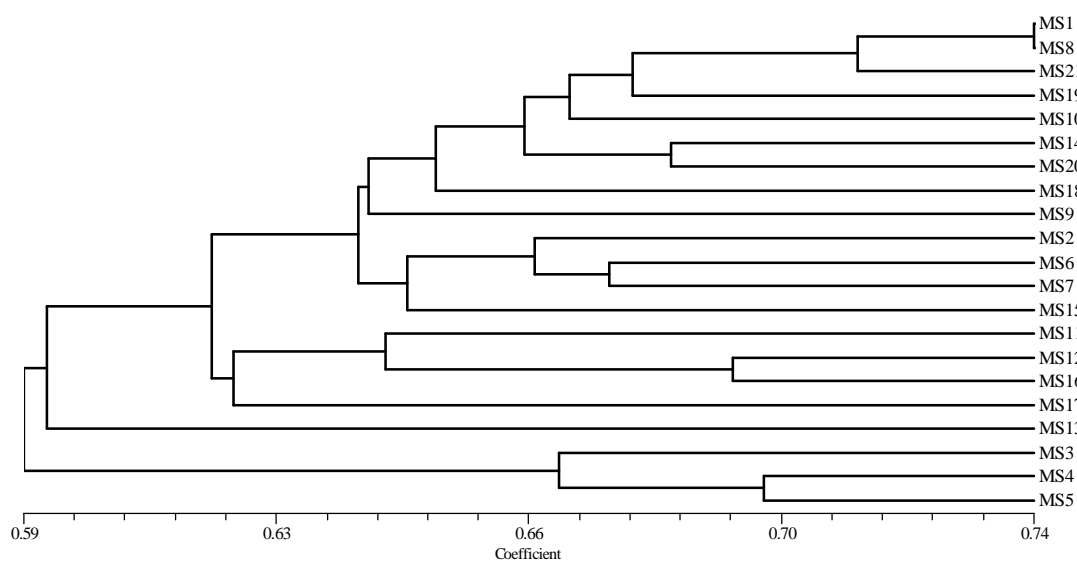




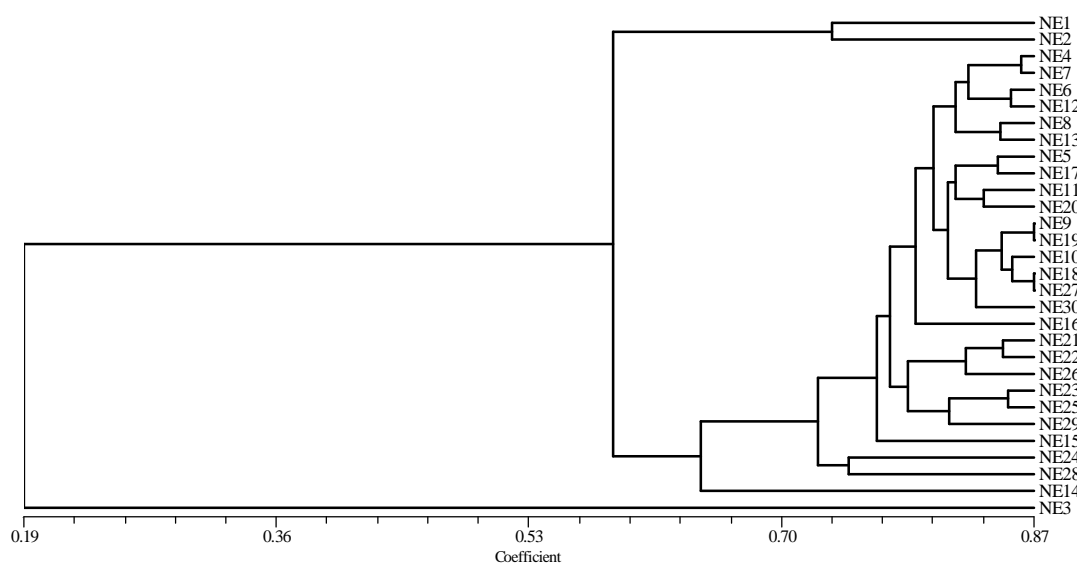
**Figure 36.** Dendrogram showing the interpopulation relationships of FAW sample collected from Hartley, Texas.



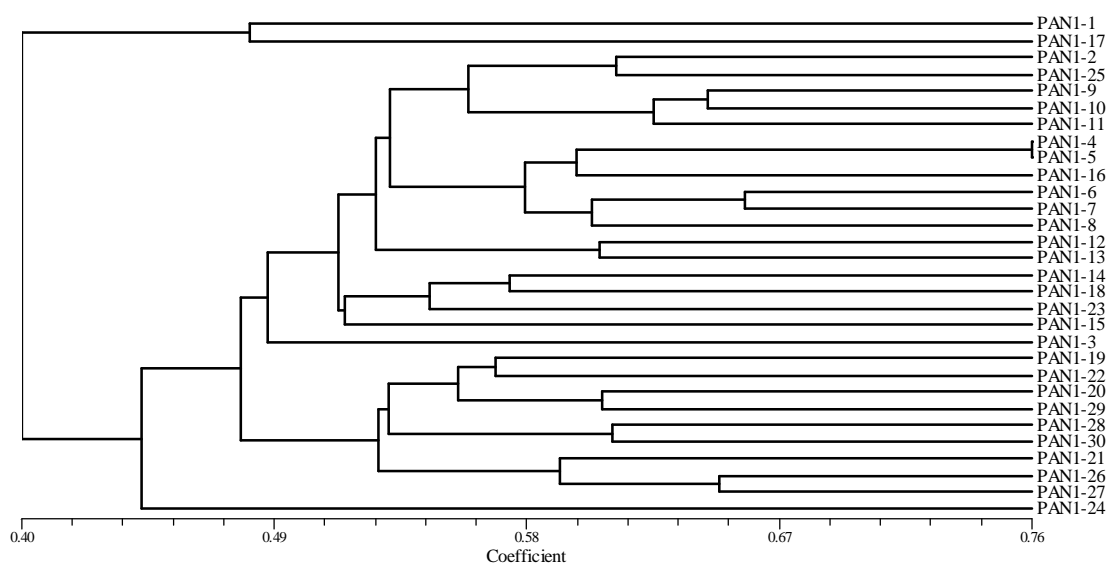
**Figure 37.** Dendrogram showing the interpopulation relationships of FAW sample collected from St. Joseph, Lusiana.



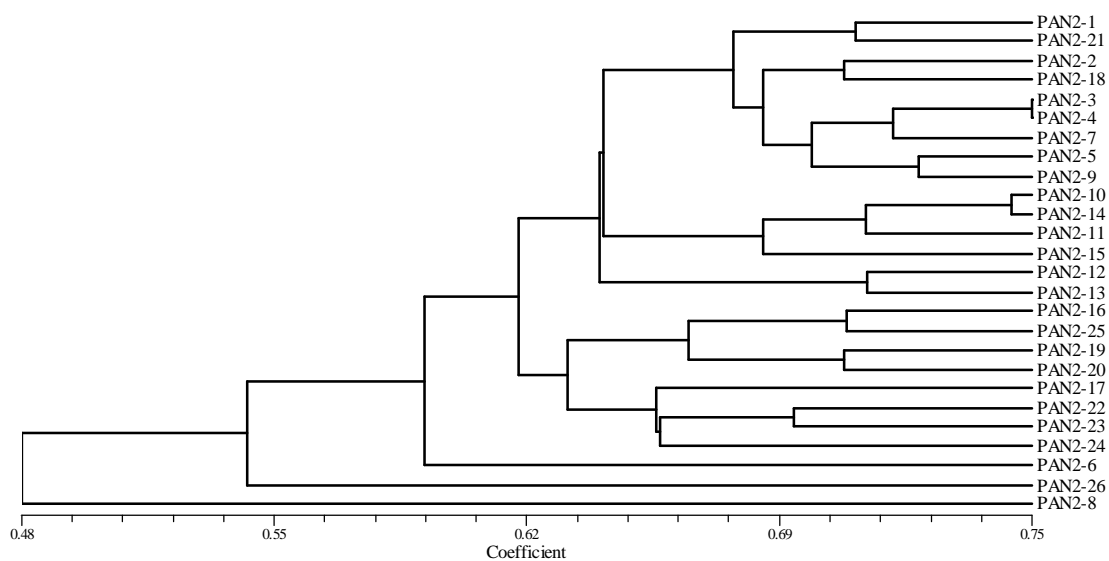
**Figure 38.** Dendrogram showing the interpopulation relationships of FAW sample collected from Washington country, Mississippi.



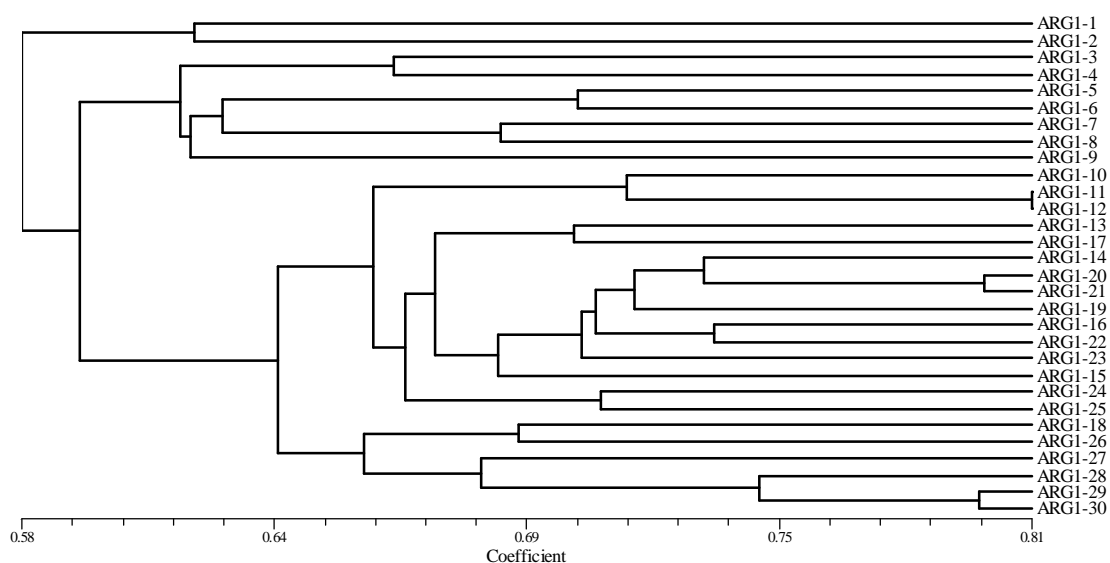
**Figure 39.** Dendrogram showing the interpopulation relationships of FAW sample collected from Elkhorn, Nebraska.



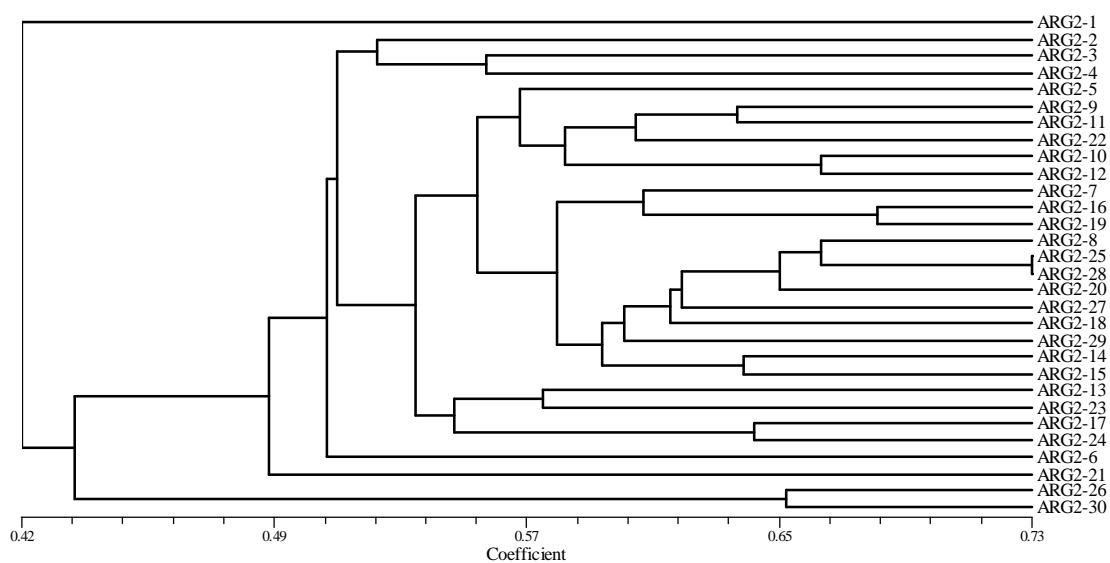
**Figure 40.** Dendrogram showing the interpopulation relationships of FAW sample collected from Pacora, Panama.



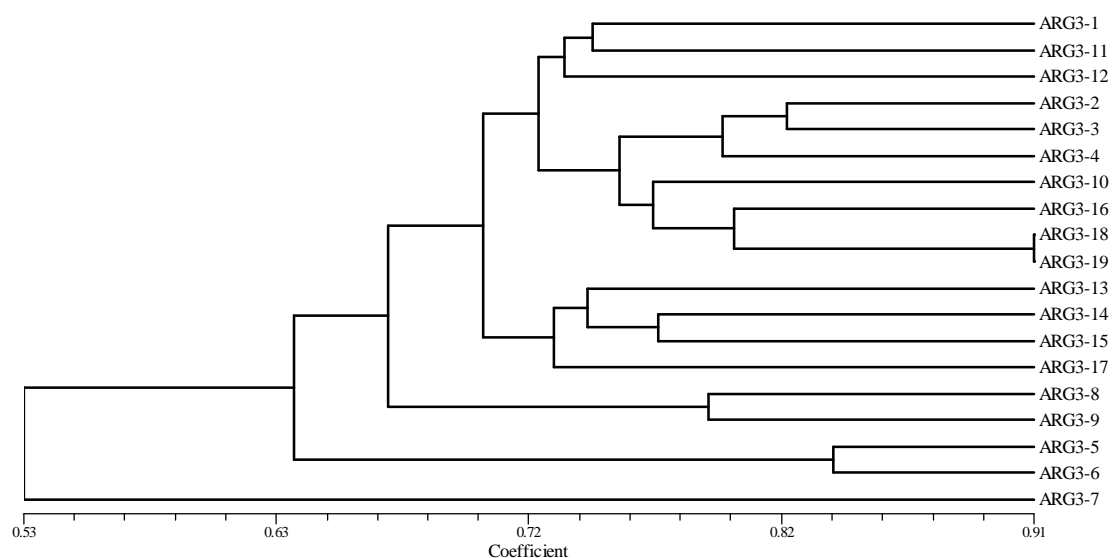
**Figure 41.** Dendrogram showing the interpopulation relationships of FAW sample collected from Chepo Panama.



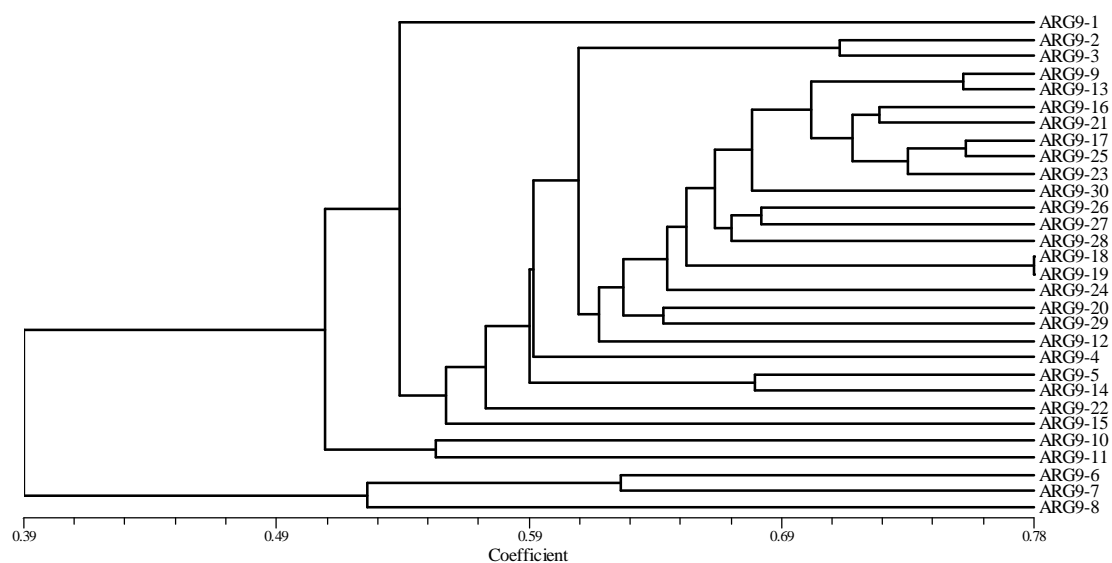
**Figure 42.** Dendrogram showing the interpopulation relationships of FAW sample collected from Alata Garacia, Argentina.



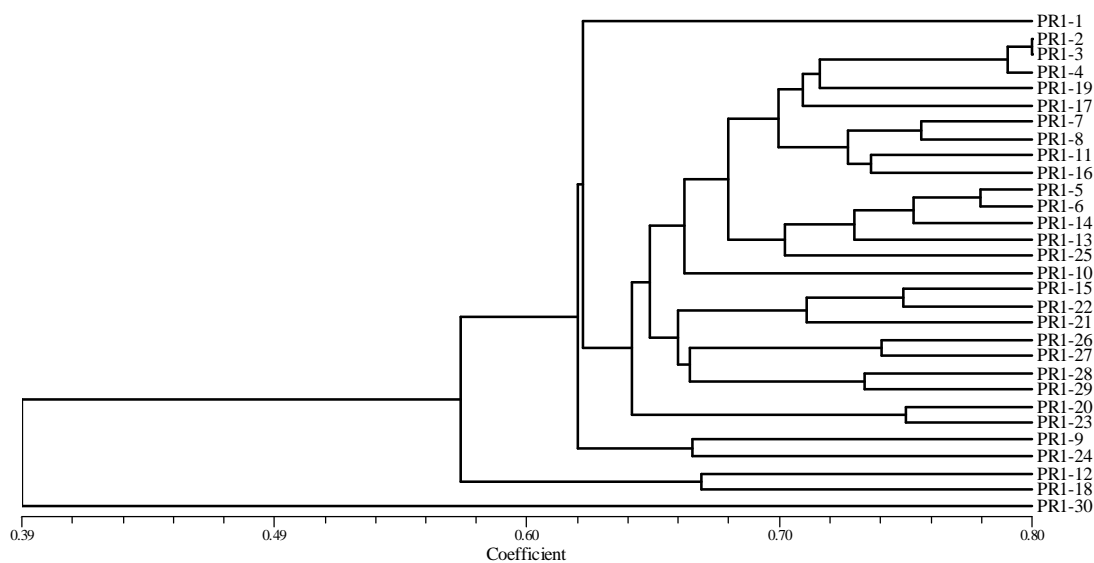
**Figure 43.** Dendrogram showing the interpopulation relationships of FAW sample collected from Canada Luque, Argentina.



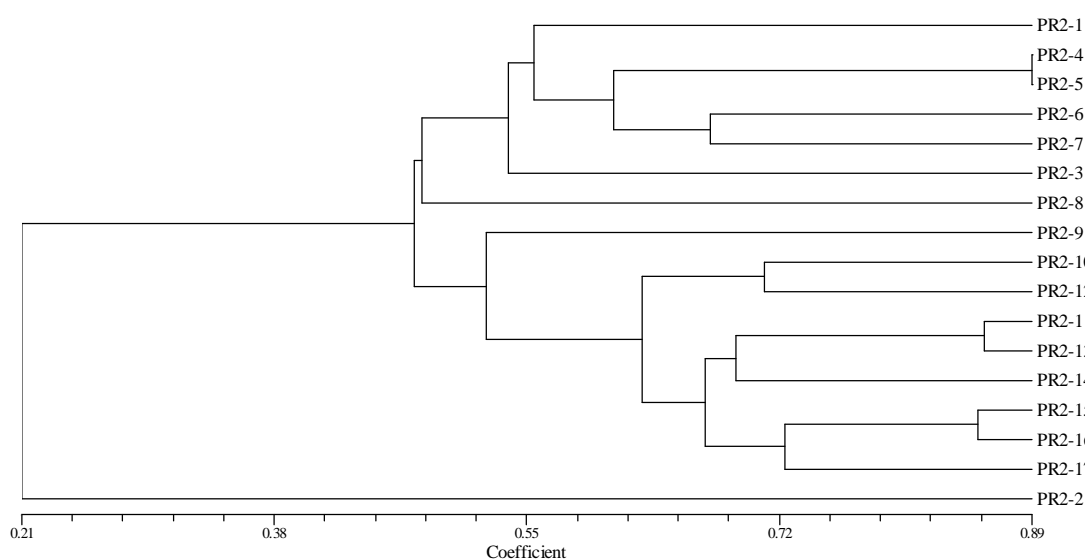
**Figure 44.** Dendrogram showing the interpopulation relationships of FAW sample collected from Tadil/BS,A.S., Argentina.



**Figure 45.** Dendrogram showing the interpopulation relationships of FAW sample collected from Fraga/Santa Fe, Argentina.



**Figure46.** Dendrogram showing the interpopulation relationships of FAW sample collected from Santa Isabel Puerto Rico.



**Figure 47.** Dendrogram showing the interpopulation relationships of FAW sample collected from Isabella, Puerto Rico.

**Declaration**

I, Difabachew Belay Kondidie hereby declare that, the research work in this Ph.D. dissertation is my own original work and has not been submitted for a degree in any other university. I have only used materials cited in the dissertation.

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May2011